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Award Number: DAMD17-99-1-9168

TITLE: New Triterpenoids for Prevention of Breast Cancer

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REPORT DATE: July 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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20030220 070

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2002	Final (1 Jun 99 - 31 May 02)

4. TITLE AND SUBTITLE
New Triterpenoids for Prevention of Breast Cancer

5. FUNDING NUMBERS
DAMD17-99-1-9168

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Original contains color plates. All DTIC reproductions will be in black and white.

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

We have shown that the synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) is a highly potent inhibitor of the proliferation of several ER-positive and ER-negative human breast cancer cell lines. Furthermore, CDDO at nanomolar levels will block de novo synthesis of two inflammatory enzymes that have been implicated in the carcinogenic process, namely inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2). During the period of funding of this grant, we have attempted to use CDDO in vivo to prevent experimental breast cancer in rats, but we have not been successful thus far, in spite of the extremely high potency of CDDO as a suppressor of the growth of numerous breast cancer cells in culture. We attribute this poor activity in vivo to inadequate pharmacokinetic properties, so therefore we have undertaken the synthesis of a large number of derivatives of CDDO, which are esters, amides, and imidazolides, relatively easily made from CDDO itself. We have evaluated many of these new structures in cell culture; they are highly active as suppressors of synthesis of iNOS and as inhibitors of growth of many tumor cells in culture, including human breast cancer cells. Evaluation of these new agents in vivo is a future goal.

14. SUBJECT TERMS	15. NUMBER OF PAGES
breast cancer	63
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

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N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Michael B. Spom 7/31/02

PI - Signature Date

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Introduction

The principal objective of this project has been an attempt to show, for the first time, that a synthetic triterpenoid can be used for the prevention of breast cancer in a valid animal model of the human disease. The eventual goal is to extend the use of a synthetic triterpenoid to prevent breast cancer in women at high risk. There is a major need for innovative drug discovery in the field of breast cancer, and a particular need for development of new agents which will inhibit progression of premalignant or early malignant lesions to more invasive and metastatic stages, since genetic and other screening techniques are now identifying large numbers of women who are at high risk for eventual development of invasive breast cancer. At the laboratory bench, this project has centered on the use of the new synthetic triterpenoid, 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO), for cancer prevention.

Body

New Triterpenoids and Prevention of Breast Cancer

Michael B. Sporn, Principal Investigator

A first major effort in this project has been the perfection of the synthesis of the triterpenoid, CDDO, and the requisite chemical methods that will allow synthesis of large amounts of material that are needed for *in vivo* studies of cancer prevention and treatment. This work has been successfully accomplished with the definitive publication of 2 full length articles in the *Journal of Medicinal Chemistry.* These articles are: Honda, T., Gribble, G. W., Suh, N., Finlay, H. J., Rounds, B. V., Bore, L., Favaloro, F. G., Wang, Y., and Sporn, M. B., "Novel synthetic oleanane and ursane triterpenoids with various enone functionalities in ring A as inhibitors of nitric oxide production in mouse macrophages." (J. Med. Chem. 43:1866-1877, 2000); and Honda, T., Rounds, B. V., Bore, L., Finlay, H. J., Favaloro, F. G., Jr., Suh, N., Wang, Y., Sporn, M. B., and Gribble, G. W., "Synthetic oleanane and ursane triterpenoids with modified rings A and C: A series of highly active inhibitors of nitric oxide production in mouse macrophages" (J. Med. Chem., 43: 4233-4246, 2000). Full details of the 11 steps that are required for the synthesis of CDDO from the starting material, oleanolic acid, are given in these articles. Support from this grant is acknowledged in both articles. We attribute this poor activity *in vivo* to inadequate pharmacokinetic properties, so therefore we have undertaken the synthesis of a large number of derivatives of CDDO, which are esters, amides, and imidazolides, relatively easily made from CDDO itself. We have evaluated many of these new structures in cell culture; they are highly active as suppressors of synthesis of iNOS and as inhibitors of growth of many tumor cells in culture, including human breast cancer cells. Details have been published (Honda et al., 2002).

The second major effort that has been undertaken in this project has been to try to prevent breast cancer in an experimental rat model. We have begun this using the classic model which employs the carcinogen, nitrosomethylurea (NMU) to initiate carcinogenesis. We have performed a series of studies giving varying doses of CDDO to the rats after initiation of carcinogenesis, and these have been uniformly unsuccessful. Details of these experiments are attached in the Appendix. We have also done some studies with this model, in which we have used combinations of CDDO, together with the rexinoid, LG100268, and in this case, there is definitely a synergistic interaction of the two agents, with enhancement of the anti-carcinogenic activity of both. Details of these experiments are presented in the Appendix. See Protocols DMS-TP-4 and DMS-TP-5. As noted in our original proposal, we have attempted to modify the classical NMU protocol developed by Thompson et al. (1995, 1998), to generate ER-negative breast cancers. In the Thompson protocols, rats were ovariectomized shortly after injection with NMU, and he reported that a substantial number of rats so treated eventually developed ER-negative breast cancers, but in our studies, we never were able to reproduce these results, so therefore we do not have any data on chemoprevention of ER-negative breast cancer by any agent in a rat. Because of the importance of ER-negative breast cancer and as an alternative, we have therefore recently turned to several ER-negative breast cancer models in transgenic mice. In these models, the mice spontaneously develop ER-negative tumors. We have recently purchased such mice, and will be starting future studies on these mice very shortly, but it is not possible to report any results at the present time.

A third major project has been to test CDDO in combination with PPAR-γ ligands and RXR ligands for inhibition of growth of ER-negative breast cancer cells in culture. We have performed such studies in a series of ER-negative breast cancer cells (21NT, 21MT-1, 21MT-2, and 21PT), all obtained from Dr. Vimla Band, Dana-Farber Cancer Institute, Boston. In these cells, we show substantial synergy of CDDO with the PPAR-γ ligand, GW7845, and the RXR ligand (rexinoid), LG100268, as documented in detail in 6 charts included in the Appendix.

Key Research Accomplishments

- Establishment of detailed and reliable synthetic procedure for synthesis of CDDO
- Synthesis of new ester, amide, and imidazolide derivatives of CDDO
- Demonstration of synergy of CDDO with the rexinoid, LG100268, in prevention of experimental breast cancer in the rat
- Demonstration of synergy of CDDO with the rexinoid, LG100268, and the PPAR-γ ligand, GW7845, in suppression of growth of human ER-negative breast cancer cells in culture

Reportable Outcomes

Published Manuscripts:

- "A new ligand for a peroxisome proliferator activated receptor-γ (PPAR-γ), GW7845, inhibits rat mammary carcinogenesis." Suh, N., Wang, Y., Williams, C. R., Risingsong, R., Gilmer, T., Willson, T. M., and Sporn, M. B. Cancer Res. 59: 5671-5673, 1999.

- "Novel synthetic oleanane and ursane triterpenoids with various enone functionalities in ring A as inhibitors of nitric oxide production in mouse macrophages."Honda, T., Gribble, G. W., Suh, N., Finlay, H. J., Rounds, B. V., Bore, L., Favaloro, F. G., Wang, Y., and Sporn, M. B. J. Med. Chem., 43:1866-1877, 2000.

- "Synthetic oleanane and ursane triterpenoids with modified rings A and C: A series of highly active inhibitors of nitric oxide production in mouse macrophages." Honda, T., Rounds, B. V., Bore, L., Finlay, H. J., Favaloro, F. G., Jr., Suh, N., Wang, Y., Sporn, M. B., and Gribble, G. W. J. Med. Chem., 43: 4233-4246, 2000.

- "Prospects for prevention and treatment of cancer with SPARMS (selective PPARγ modulators)." Sporn, M. B., Suh, N., and Mangelsdorf, D. J. Trends Mol. Med., 7: 395-400, 2001.

- "A novel dicyanotriterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile, active at picomolar concentrations for inhibition of nitric oxide production." Honda, T., Honda, Y., Favaloro, F. G., Gribble, G. W., Suh, N., Place, A. E., Rendi, M. H., Sporn, M. B. Bioorganic & Medicinal Chemistry Letters: 12: 1027-1030, 2002.

- "Chemoprevention: an essential approach to control cancer." Sporn, M. B., Suh, N. Nature Rev. Cancer 2: 537-543, 2002.

Conclusions

Synthetic triterpenoids represent an important class of new drugs that have potential for clinical use for prevention and treatment of breast cancer. However, a great deal more research will need to be done before this will be clinically practical. In particular, a whole new set of pharmacokinetic studies will need to be done in the future.

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- Honda, T., Honda, Y., Favaloro, F. G., Gribble, G. W., Suh, N., Place, A. E., Rendi, M. H., Sporn, M. B. A novel dicyanotriterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile, active at picomolar concentrations for inhibition of nitric oxide production. Bioorganic & Medicinal Chemistry Letters: 12: 1027-1030, 2002.
- Honda, T., Rounds, B. V., Bore, L., Finlay, H. J., Favaloro, F. G., Jr., Suh, N., Wang, Y., Sporn, M. B., and Gribble, G. W., Synthetic oleanane and ursane triterpenoids with modified rings A and C: A series of highly active inhibitors of nitric oxide production in mouse macrophages. J. Med. Chem., 43: 4233-4246, 2000.
- Sporn, M. B., Suh, N. Chemoprevention: an essential approach to control cancer. Nature Rev. Cancer 2: 537-543, 2002.
- Sporn, M. B., Suh, N., and Mangelsdorf, D. J. Prospects for prevention and treatment of cancer with SPARMS (selective PPARγ modulators). Trends Mol. Med., 7: 395-400, 2001.
- Suh, N., Wang, Y., Williams, C. R., Risingsong, R., Gilmer, T., Willson, T. M., and Sporn, M. B.: A new ligand for a peroxisome proliferator activated receptor-γ (PPAR-γ), GW7845, inhibits rat mammary carcinogenesis. Cancer Res. 59: 5671-5673, 1999.
- Thompson, H.J., McGInley, J., Rothammer, K., and Singh, M. Ovarian hormone dependence of pre-malignant mammary gland lesions induced in pre-pubertal rats by 1-methyl-1-nitrosourea. Carcinogenesis, 19: 383-386, 1998.
- Thompson, H.J., McGInley, J.N., Rothammer, K., and Singh, M. Rapid induction of mammary intraductal proliferations, ductal carcinoma *in situ* and carcinomas by the injection of sexually immatrue female rats with 1-methyl-1-nitrosourea. Carcinogenesis, 16: 2407-2411, 1995.

List of Personnel Receiving Pay from this Grant

- Michael B. Sporn, M.D.
- Tadashi Honda, Ph.D.
- Nanjoo Suh, Ph.D.
- Renee Risingsong, B.S.
- Charlotte Williams, B.A.

Appendices

Appendix 1: Figures and charts

Appendix 2: "A new ligand for a peroxisome proliferator activated receptor-γ (PPAR-γ), GW7845, inhibits rat mammary carcinogenesis." Suh, N., Wang, Y., Williams, C. R., Risingsong, R., Gilmer, T., Willson, T. M., and Sporn, M. B. Cancer Res. 59: 5671-5673, 1999.

Appendix 3: "Novel synthetic oleanane and ursane triterpenoids with various enone functionalities in ring A as inhibitors of nitric oxide production in mouse macrophages."Honda, T., Gribble, G. W., Suh, N., Finlay, H. J., Rounds, B. V., Bore, L., Favaloro, F. G., Wang, Y., and Sporn, M. B. J. Med. Chem., 43:1866-1877, 2000.

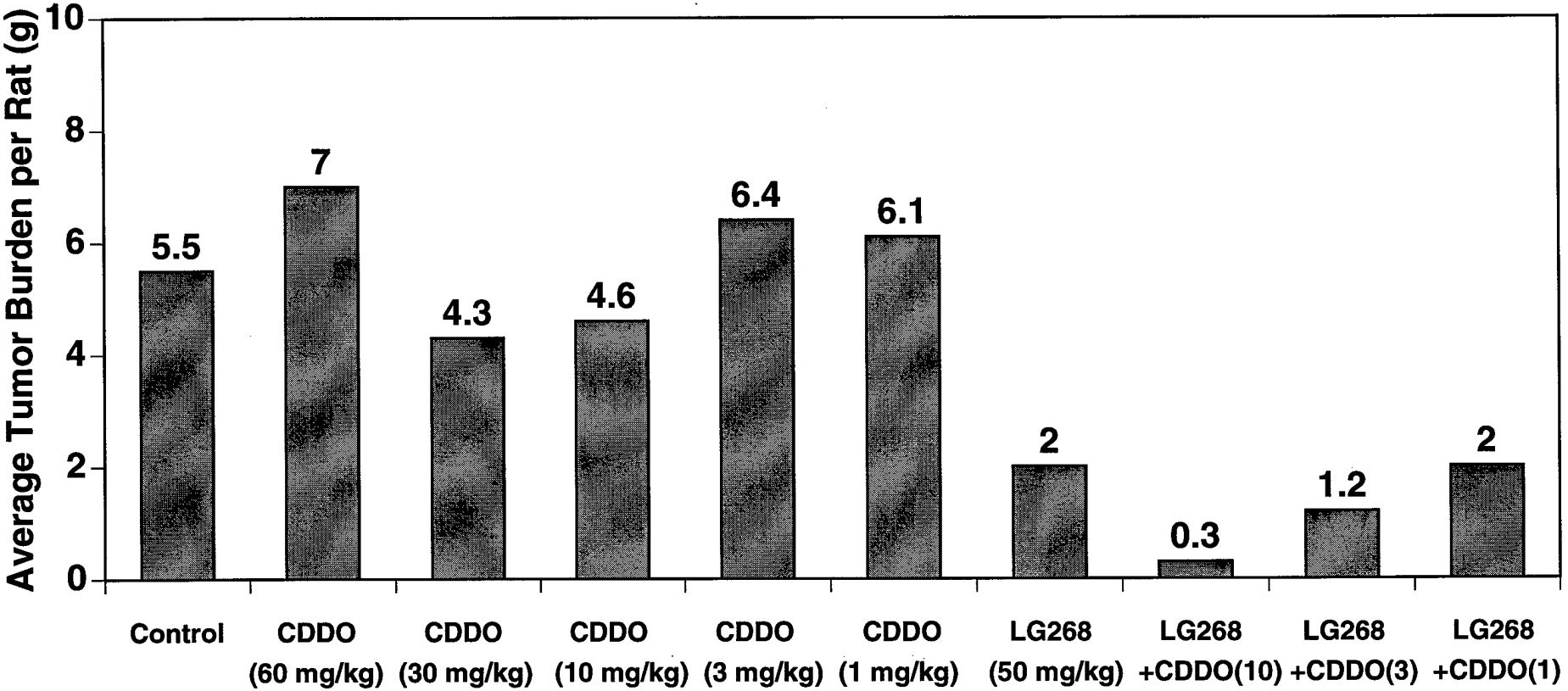
Appendix 4: "Synthetic oleanane and ursane triterpenoids with modified rings A and C: A series of highly active inhibitors of nitric oxide production in mouse macrophages." Honda, T., Rounds, B. V., Bore, L., Finlay, H. J., Favaloro, F. G., Jr., Suh, N., Wang, Y., Sporn, M. B., and Gribble, G. W. J. Med. Chem., 43: 4233-4246, 2000.

Appendix 5:"Prospects for prevention and treatment of cancer with SPARMS (selective PPARγ modulators)." Sporn, M. B., Suh, N., and Mangelsdorf, D. J. Trends Mol. Med., 7: 395-400, 2001.

Appendix 6: "A novel dicyanotriterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile, active at picomolar concentrations for inhibition of nitric oxide production." Honda, T., Honda, Y., Favaloro, F. G., Gribble, G. W., Suh, N., Place, A. E., Rendi, M. H., Sporn, M. B. Bioorganic & Medicinal Chemistry Letters: 12: 1027-1030, 2002.

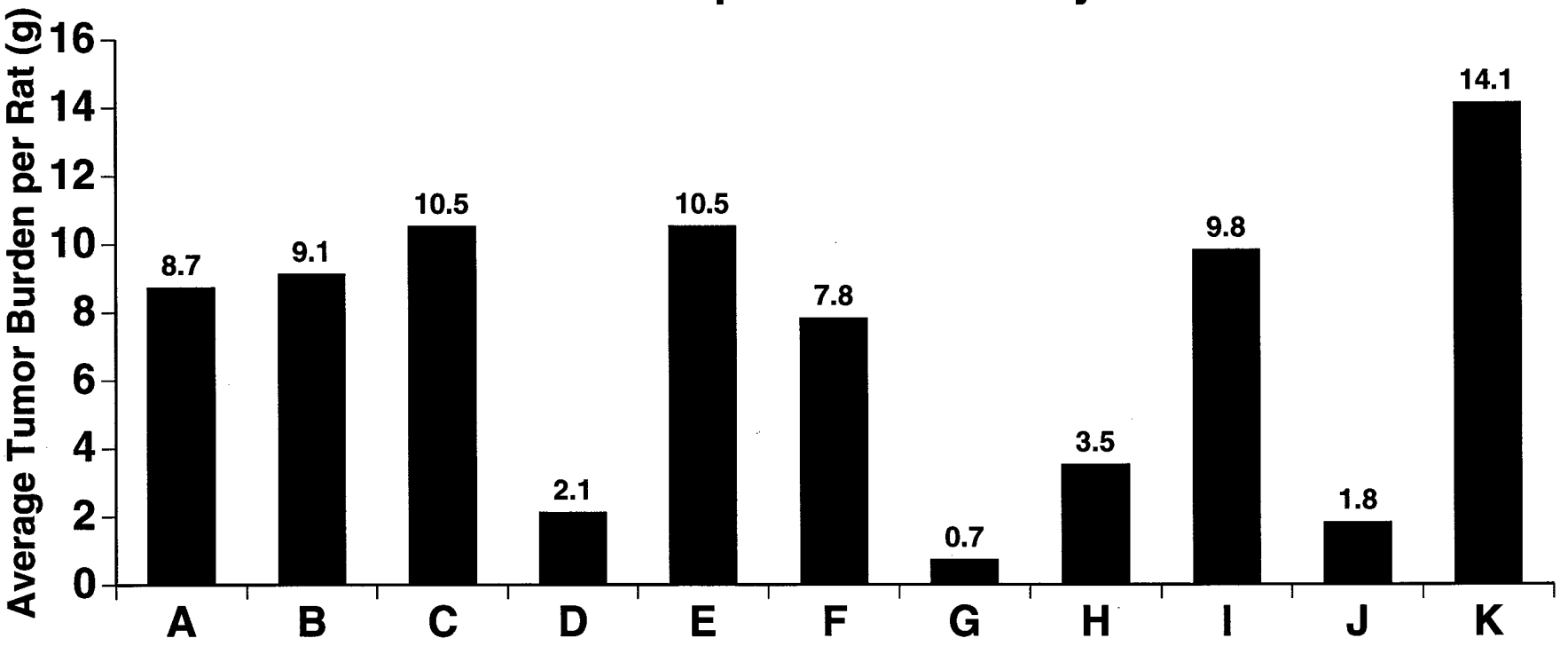
Appendix 7: "Chemoprevention: an essential approach to control cancer." Sporn, M. B., Suh, N. Nature Rev. Cancer 2: 537-543, 2002.

Protocol DMS-TP-4
Breast Cancer Chemoprevention Study in Rats
(Average Tumor Burden at Autopsy)



*Rats were autopsied at 7 weeks after NMU injection.

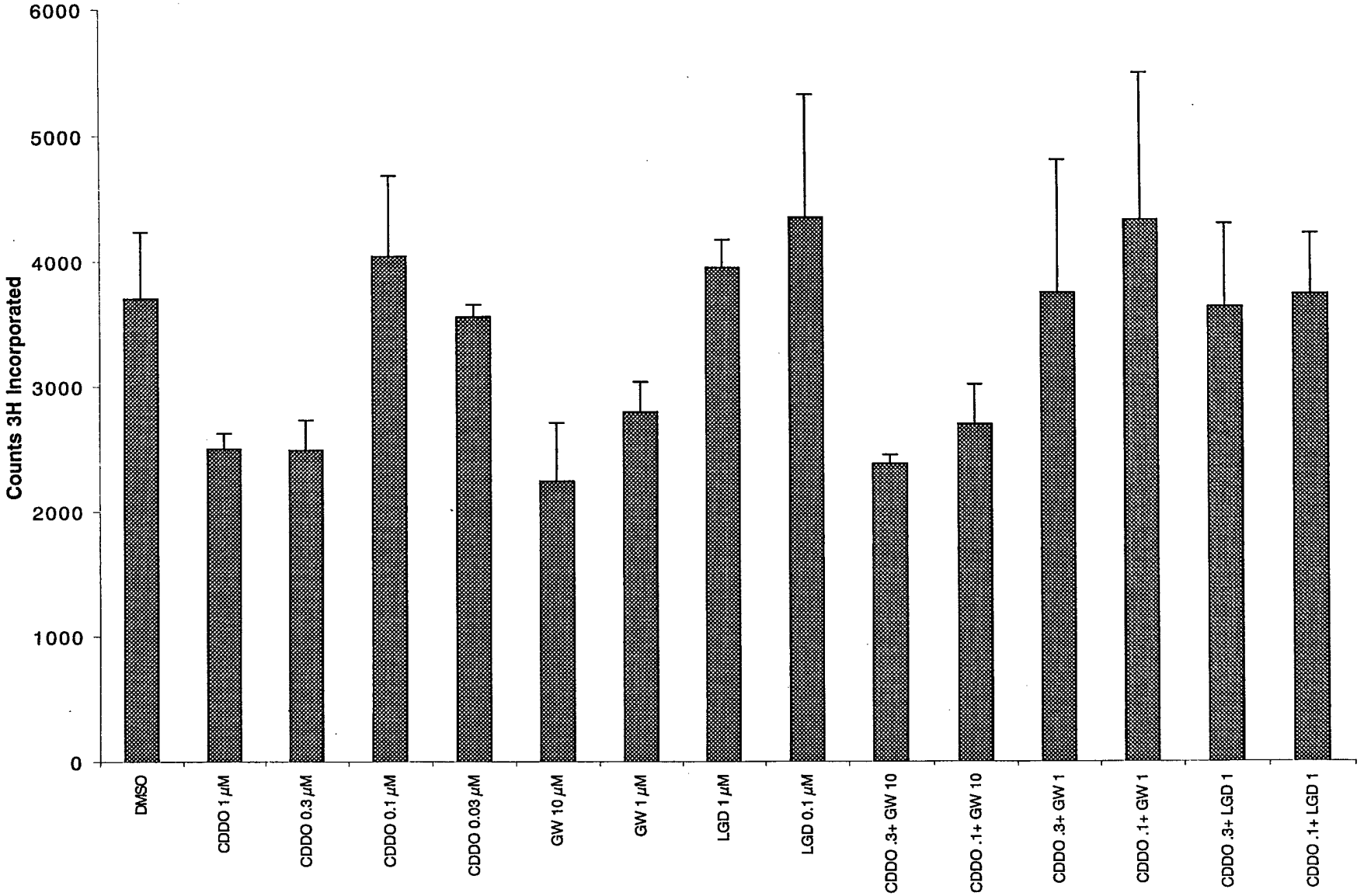
Protocol DMS-TP-5:
Breast Cancer Chemoprevention Study in Rats



	A	B	C	D	E	F	G	H	I	J	K
CDDO	-	30	10	-	-	-	30	30	30	10	10
LG100268	-	-	-	60	-	-	60	-	-	60	-
9-cis-RA	-	-	-	-	60	-	-	60	-	-	60
At-RA	-	-	-	-	-	60	-	-	60	-	-
(mg/kg diet)											

Effects of CDDO, GW, and LGD Compounds on Growth of 21-NT ER-Negative Cells

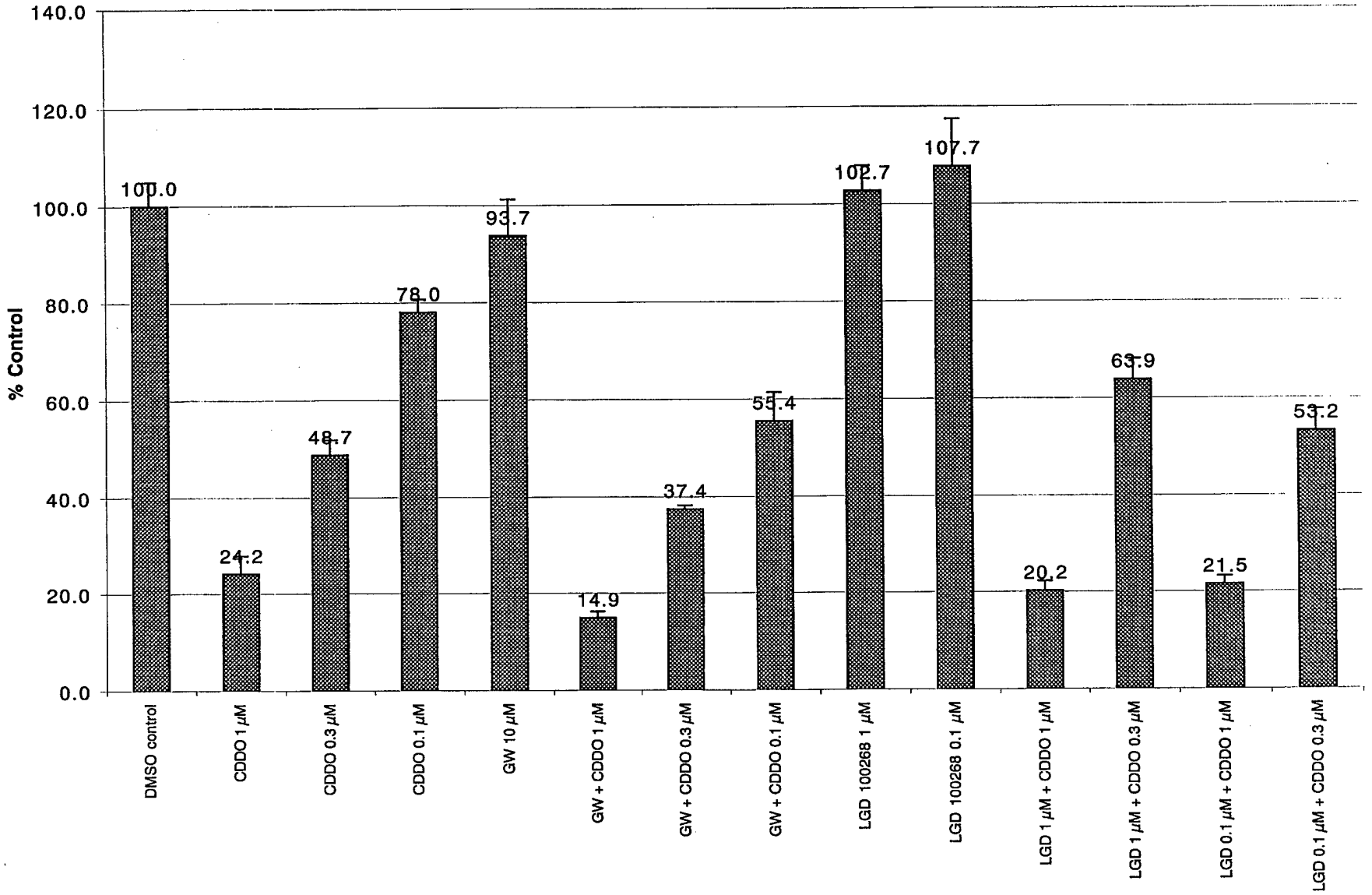
(7845) (268)



Three days incubation in compounds, 10% FBS growth media
Two hours thymidine pulse

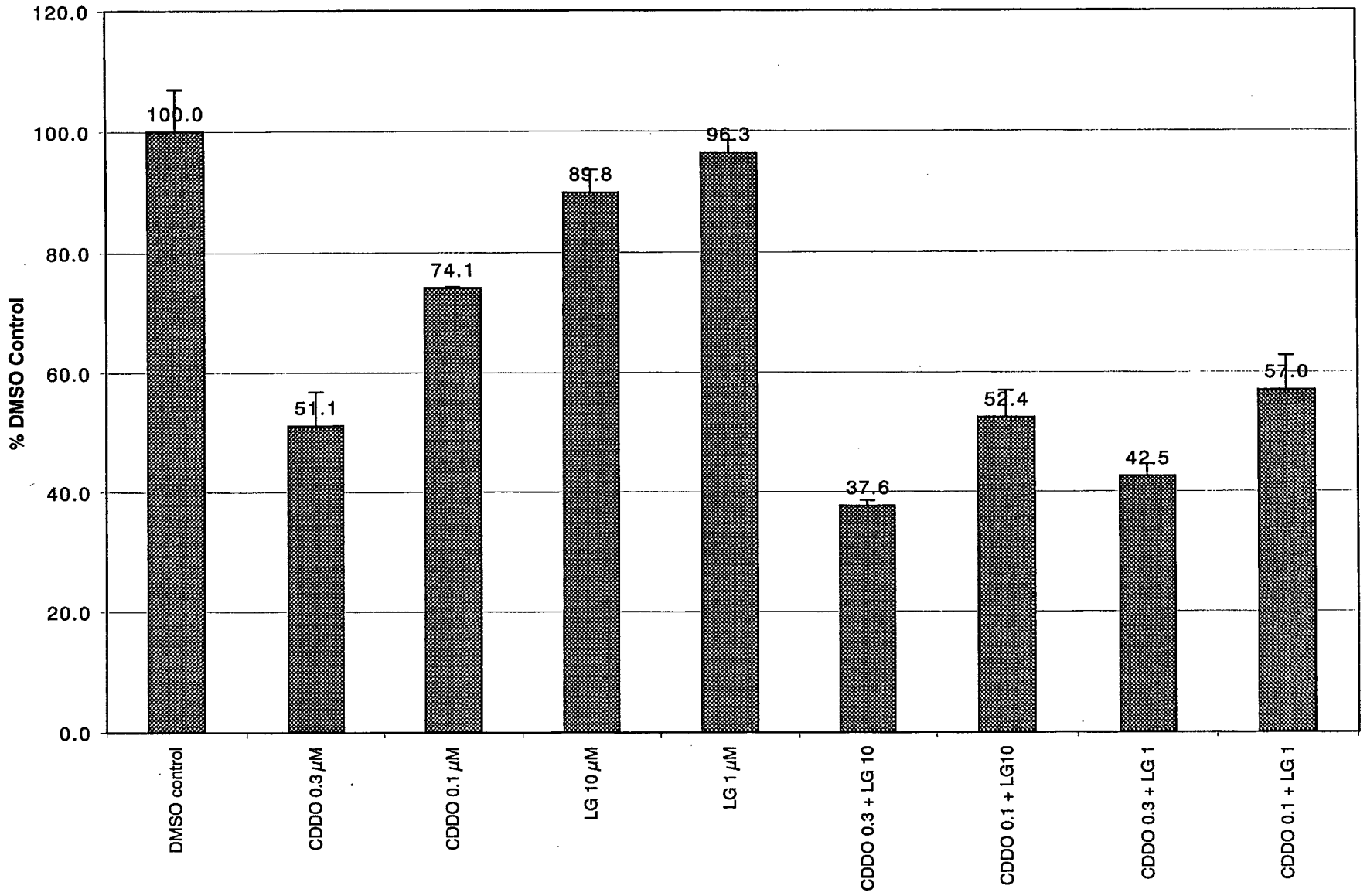
Combination Effects of CDDO, GW and LGD Compounds on Growth of 21MT-1 ER Negative Breast Cancer Cells

(7845) (268)



Three days incubation with compounds in 10% FBS growth media
Two hours thymidine pulse

Synergy Between CDDO and LGD 100268 in Growth Inhibition of 21 MT-1 Breast Cancer Cells

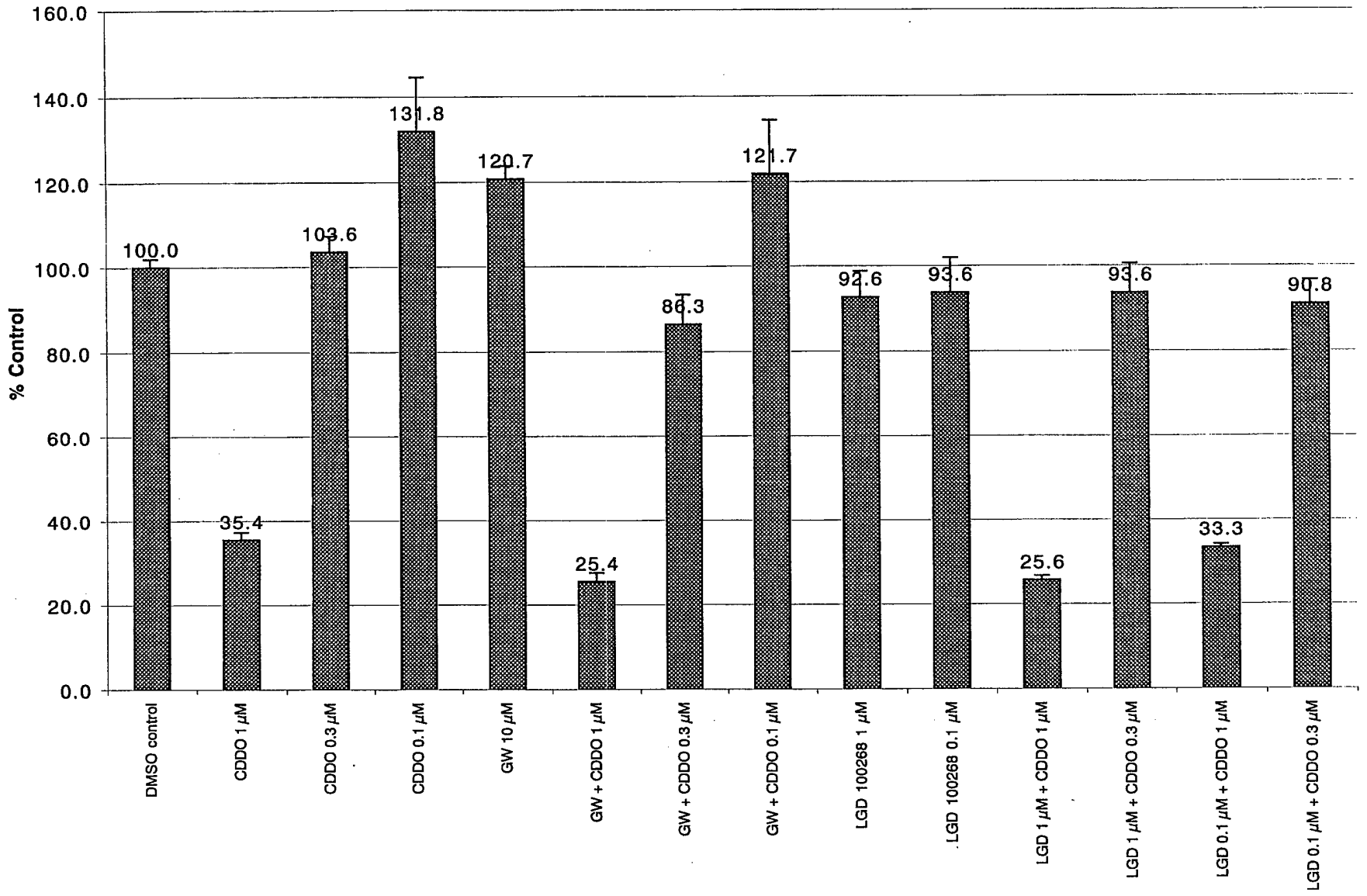


72 hours incubation with compounds in growth media
2 hours thymidine pulse

[redacted]

Combination Effects of CDDO, GW and LGD Compounds on Growth of 21MT-2 ER Negative Breast Cancer Cells

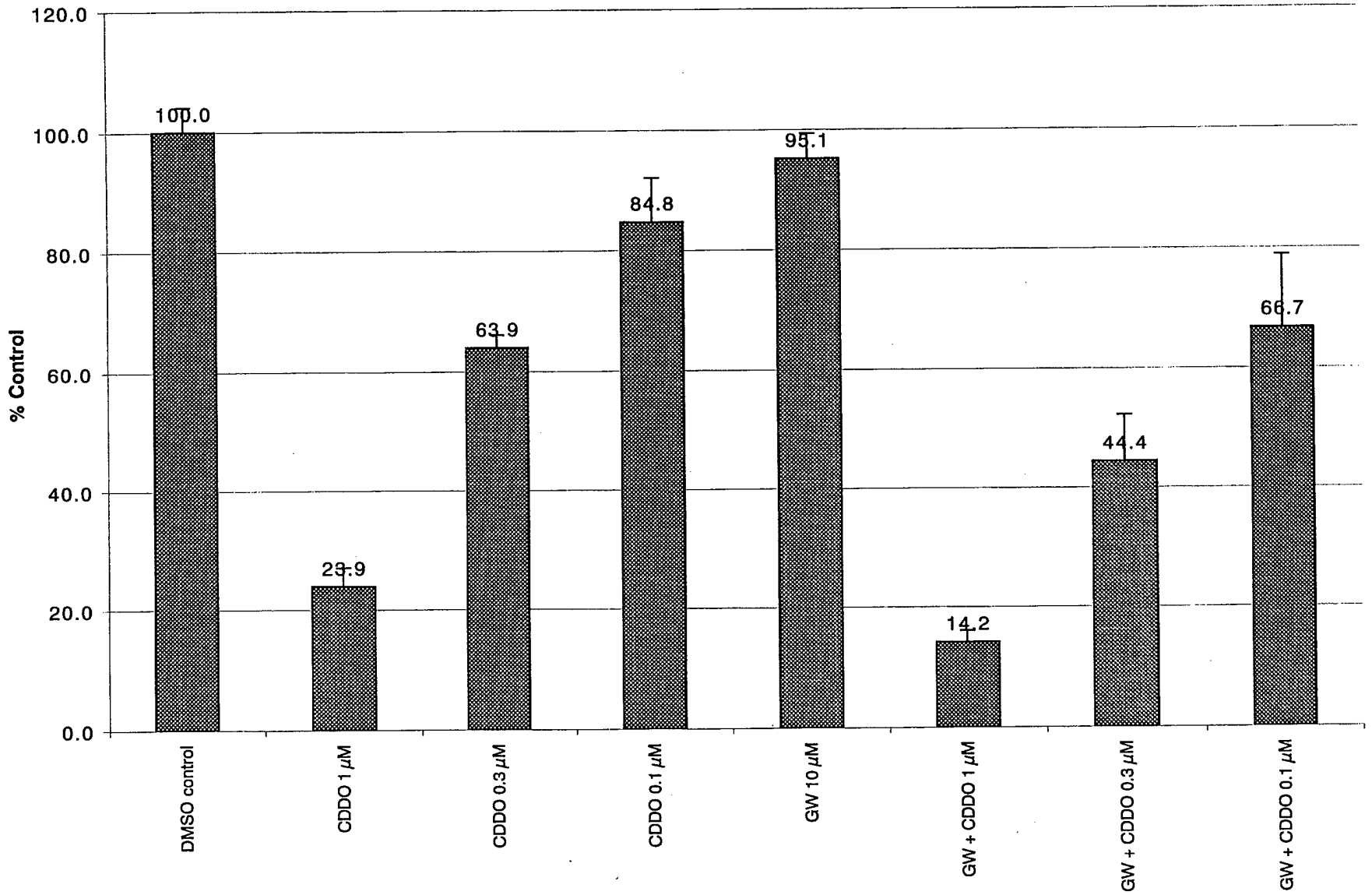
(7845) (268)



Three days incubation with compounds in 10% FBS growth media
Two hours thymidine pulse

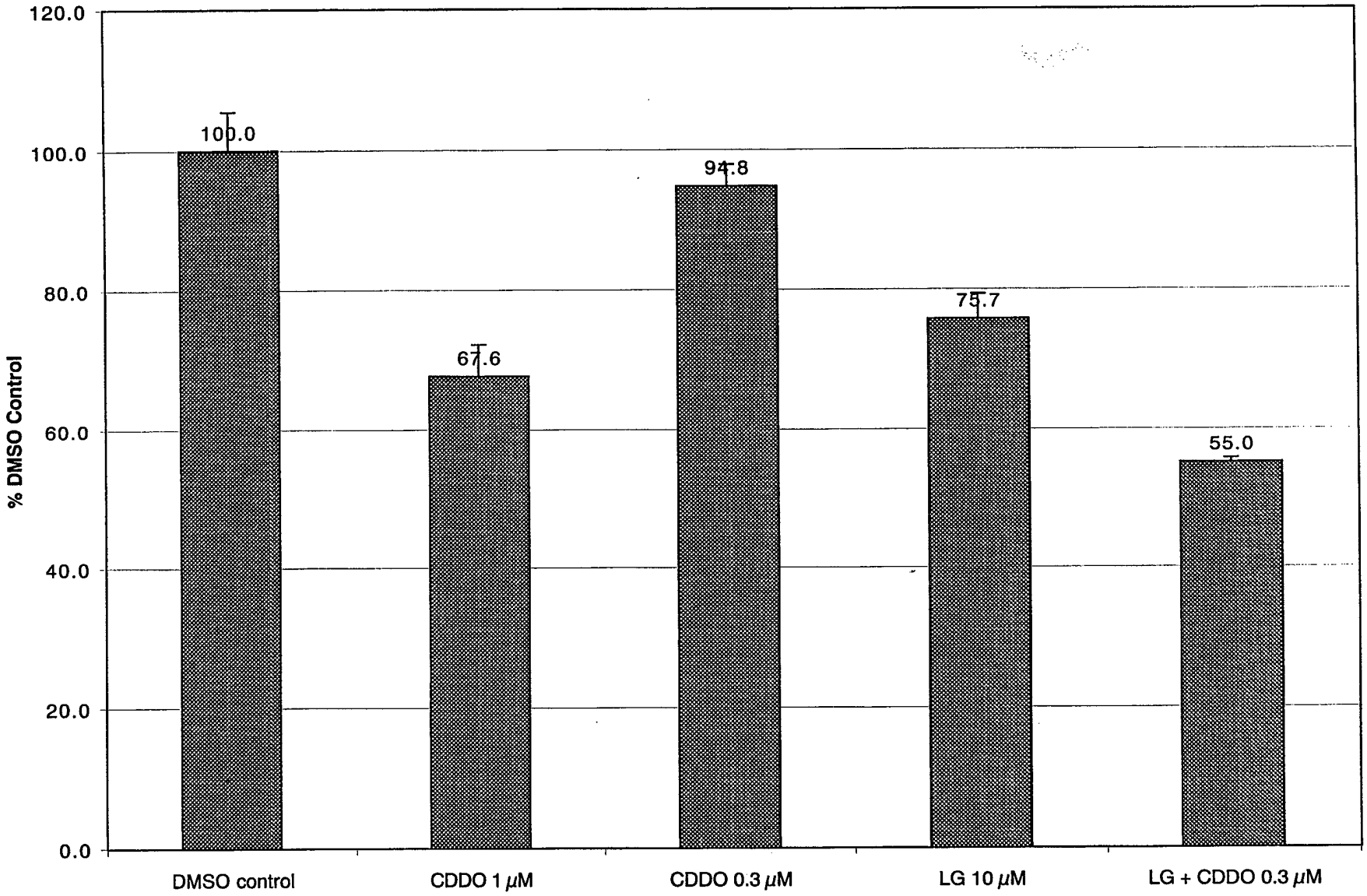
CDDO and GW Compound Synergize in Growth Inhibition of 21PT ER Negative Breast Cancer Cells

(7845)



Three days incubation with compounds in 10% FBS growth media
Two hours thymidine pulse

Synergy Between CDDO and LGD100268 in Growth Inhibition of 21PT Breast Cancer Cells



72 hours incubation with compounds in growth media
2 hours thymidine pulse

[redacted]

[CANCER RESEARCH 59, 5671–5673, November 15, 1999]

Advances in Brief

A New Ligand for the Peroxisome Proliferator-Activated Receptor-γ (PPAR-γ), GW7845, Inhibits Rat Mammary Carcinogenesis[1]

Nanjoo Suh, Yongping Wang, Charlotte R. Williams, Renee Risingsong, Tona Gilmer, Timothy M. Willson, and Michael B. Sporn[2]

Department of Pharmacology and Norris Cotton Cancer Center, Dartmouth Medical School, Hanover, New Hampshire 03755 [N. S., Y. W., C. R. W., R. R., M. B. S.], Glaxo Wellcome Research and Development, Research Triangle Park, North Carolina 27709 [T. G., T. M. W.]

Abstract

We have tested a new ligand for peroxisome proliferator-activated receptor-γ, GW7845, as an inhibitor of experimental mammary carcinogenesis, using the classic rat model with nitrosomethylurea as carcinogen. Rats were first treated with a single dose of nitrosomethylurea (50 mg/kg body weight, i.p.). Starting 1 week later, they were fed GW7845, at either 60 or 30 mg/kg of diet, for 2 months. This agent significantly reduced tumor incidence, tumor number, and tumor weight at both doses. This is the first report of the use of a ligand for peroxisome proliferator-activated receptor-γ to prevent experimental breast cancer.

Introduction

The continuing magnitude of the breast cancer problem with respect to incidence, morbidity, and mortality requires further drug discovery to prevent this disease (1). The use of tamoxifen, raloxifene, and fenretinide as clinically proven, effective agents to suppress breast carcinogenesis (2–4) indicates that chemoprevention is a viable strategy for the prevention of breast cancer in women. Current research in this area is driven by the need to discover new agents that will be more effective and have fewer side effects. In this brief communication, we report the first use of a new and highly potent ligand for the nuclear receptor, PPAR-γ,[3] GW7845 to inhibit experimental mammary carcinogenesis *in vivo*.

PPAR-γ is a transcription factor belonging to the nuclear receptor superfamily (5–7) and forms functional heterodimers with the retinoid X receptor (8). PPAR-γ is of great current interest because it mediates the antidiabetic effects of several TZDs that are now in widespread clinical use for treatment of type 2 diabetes (9, 10). The PPARs bind a variety of naturally occurring fatty acids and eicosanoids with low micromolar affinity (6). Interestingly, PPAR-γ has a preference for polyunsaturated fatty acids (11), dietary components that have been shown to lower the incidence of cancer in experimental animals (12, 13), although the clinical relevance of these observations remains unclear (12, 14).

Synthetic PPAR-γ ligands have been shown to inhibit growth of several human tumor cell lines in culture (15–17) and, most notably, to induce growth arrest and differentiation in primary cultures of human liposarcoma cells, both *in vitro* and *in vivo* (18, 19). In contrast, there have been conflicting reports on the effects of the TZD class of PPAR-γ ligands in experimental colon carcinogenesis (20–22). The mechanism of inhibition of growth of tumor cells by ligands for PPAR-γ is not well understood (23). For the present study, reported here, the availability of a potent member of a new class of ligands for PPAR-γ, GW7845 (24), has enabled us to test this agent for inhibition of mammary carcinogenesis in the classic rat model that uses NMU as carcinogen.

Materials and Methods

Cell Culture and Differentiation Assays. GW7845 was dissolved in DMSO (0.01 M), and aliquots were frozen at −20°C. Serial dilutions were made in DMSO before addition to cell culture media. The 3T3-L1 preadipocyte cells were obtained from American Type Culture Collection, grown to confluency in DMEM/5% calf serum, and then treated once with compounds in DMEM/10% fetal bovine serum. Every 2 days thereafter, medium was changed to DMEM/10% fetal bovine serum without added compounds. Cells were harvested on day 6, and as a marker of differentiation, glycerol 3-phosphate dehydrogenase was measured in lysates, using a standard assay for consumption of NADH at 340 nm (25).

Mammary Carcinogenesis Studies. A total of 159 female Sprague Dawley rats (Taconic Farms, Germantown, NY) received i.p. injections of NMU (50 mg/kg body weight) when 21 days old, as described by Thompson *et al.* (26). One week later, the rats were randomly assigned to one of six experimental groups (Table 1). GW7845 and tamoxifen were blended into the diets as described previously (27) and were fed to the rats continuously, either alone or in combination, for the duration of the experiment. Rats were killed after 2 months (CO_2 inhalation), and breast cancers were enumerated and weighed at autopsy.

Other. The Fisher exact test and the Mann-Whitney rank test were used to evaluate the statistical differences between the treatment groups; all P values shown are two-sided. Institutional guidelines for proper and humane use of rats were observed.

Results and Discussion

GW7845 is a tyrosine analogue (Fig. 1), rather than a TZD such as troglitazone, rosiglitazone, and pioglitazone (the ligands for PPAR-γ in current clinical use). Unlike the TZDs, GW7845 has been optimized for potency on PPAR-γ (24) and is significantly more potent than either rosiglitazone or troglitazone when assayed for induction of adipogenic differentiation in the fibroblastic cell line, 3T3-L1 (25), as shown in Fig. 2.

We have performed two separate but identical long-term experiments to demonstrate the chemopreventive efficacy of GW7845. Given the widespread use of tamoxifen as an agent to prevent breast cancer, we have also looked at potential synergism between GW7845 and tamoxifen. The results in both experiments were essentially identical; therefore, we have pooled the data in Table 1.

GW7845 was well tolerated at the doses fed (Table 1), and rats treated with this agent weighed the same as controls. Table 1 shows that GW7845 had significant inhibitory effects on mammary carcinogenesis regardless of whether tumor incidence, numbers of tumors per rat, or ATB (the average weight of a rat's tumor at autopsy) was

Received 8/23/99; accepted 10/5/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the National Foundation for Cancer Research, NIH Grant R01 CA78814, and DOD/AMRD Award 17-99-1-9168. M. B. S. is Oscar M. Cohn Professor, and Y. W. is a Howard Hughes Medical Institute predoctoral fellow.

[2] To whom requests for reprints should be addressed, at Department of Pharmacology, Dartmouth Medical School, 7650 Remsen, Hanover, NH 03755. Phone: (603) 650-6557; Fax: (603) 650-1129; E-mail: Michael.Sporn@dartmouth.edu.

[3] The abbreviations used are: PPAR-γ, peroxisome proliferator-activated receptor-γ; TZD, thiazolidinedione; NMU, nitrosomethylurea; ATB, average tumor burden.

Table 1 *Prevention of breast cancer by GW7845 and tamoxifen*

Treatment[a]	No. of tumor-free rats/total no. of rats (P_1; P_2)[b]	Average no. of tumors (P_1; P_2)[b]	ATB (P_1; P_2)[b]	Rats with 3 or more tumors (P_1; P_2)[b]	Rats with tumor burden >5 g (P_1; P_2)[b]
Control (vehicle)	5/42	2.4	5.6	22/42	18/42
GW7845 Hi	8/21 (0.02)	1.1 (0.002)	1.7 (0.002)	2/21 (0.0009)	1/21 (0.002)
GW7845 Lo	7/21 (0.08)	0.8 (<0.0001)	1.5 (0.0004)	0/21 (<0.0001)	2/21 (0.009)
Tamoxifen	5/33	1.6 (0.02)	2.4 (0.02)	7/33 (0.008)	6/33 (0.03)
Tamoxifen + GW7845 Hi	9/21 (0.009; 0.03)	0.9 (0.0002; 0.03)	0.9 (0.0002; 0.05)	0/21 (<0.0001; 0.03)	0/21 (0.0002)
Tamoxifen + GW7845 Lo	12/21 (0.0003; 0.002)	0.6 (<0.0001; 0.001)	1.3 (0.0001; 0.01)	1/21 (0.0002)	3/21 (0.03)

[a] Doses used were as follows: 60 mg GW7845/kg diet (GW7845 Hi); 30 mg GW7845/kg diet (GW7845 Lo); and 0.5 mg tamoxifen/kg diet. All animals (21 days old) received an i.p. injection of 50 mg NMU/kg body weight 1 week before starting the feeding of chemopreventive agents.

[b] P_1 is the value for the comparison of rats treated with chemopreventive agents with control rats treated with vehicle alone; P_2 is the value for the comparison of rats treated with tamoxifen + GW7845 with rats treated with tamoxifen alone.

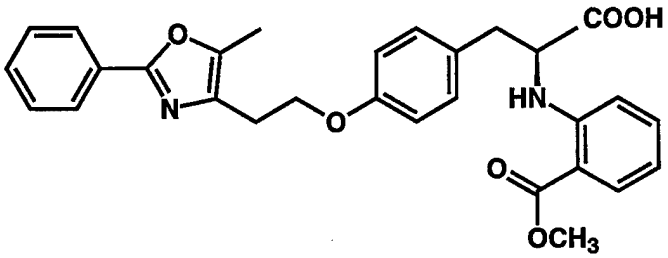


Fig. 1. Structure of GW7845.

measured. The effects on ATB are particularly interesting; GW7845 effected a 70% reduction in this index. Striking effects of GW7845 on tumor multiplicity and weight were seen (Table 1) when the number of rats with three or more tumors or the number of rats with a tumor burden >5 g were scored. Both doses of GW7845 appeared equally effective in all parameters measured. To evaluate possible synergy with tamoxifen, we deliberately chose a very low dose of this agent, which is only marginally effective (27, 28). As seen in Table 1, although some statistically significant additive effects were seen with the combination of GW7845 and tamoxifen, there was little evidence in these experiments for a strong synergy between the two.

These initial experiments *in vivo* establish GW7845 as an agent worthy of further consideration for chemoprevention of cancer. Further studies in other organ systems in which PPAR-γ plays an important role, as well as potential synergy with other agents for which there is a mechanistic basis (*e.g.*, selective ligands for the retinoid X receptor), should now be pursued, as well as further evaluation of the mechanism of suppression of carcinogenesis by PPAR-γ.

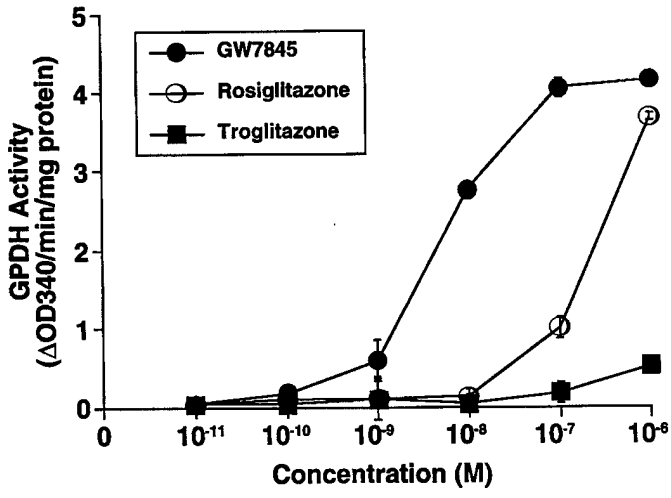


Fig. 2. GW7845 is more potent than either rosiglitazone or troglitazone in induction of adipogenic differentiation in 3T3-L1 fibroblasts. Adipogenesis was measured after 6 days of treatment, as described (25), using a glycerol 3-phosphate dehydrogenase assay as a marker. *OD340*, absorbance at 340 nm; *bars*; SE.

Acknowledgments

We thank Tammy Frazer for expert assistance in preparation of the manuscript. Marilyn Brown and her staff, especially Jennifer Marcroft and Catherine LaBarre, have provided excellent animal care.

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Novel Synthetic Oleanane and Ursane Triterpenoids with Various Enone Functionalities in Ring A as Inhibitors of Nitric Oxide Production in Mouse Macrophages†

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Received January 7, 2000

We initially randomly synthesized about 60 oleanane and ursane triterpenoids as potential anti-inflammatory and cancer chemopreventive agents. Preliminary screening of these derivatives for inhibition of production of nitric oxide induced by interferon-γ in mouse macrophages revealed that 3-oxooleana-1,12-dien-28-oic acid (**B-15**) showed significant activity (IC_{50} = 5.6 μM). On the basis of the structure of **B-15**, 19 novel olean- and urs-12-ene triterpenoids with a 1-en-3-one functionality having a substituent at C-2 in ring A have been designed and synthesized. Among them, 3-oxooleana-1,12-diene derivatives with carboxyl, methoxycarbonyl, and nitrile groups at C-2 showed higher activity than the lead compound **B-15**. In particular, 2-carboxy-3-oxooleana-1,12-dien-28-oic acid (**3**) had the highest activity (IC_{50} = 0.07 μM) in this group of triterpenoids. The potency of **3** was similar to that of hydrocortisone (IC_{50} = 0.01 μM), although **3** does not act through the glucocorticoid receptor. Interesting structure−activity relationships of these novel synthetic triterpenoids are also discussed.

Introduction

Oleanane and ursane triterpenoids are pentacyclic compounds with 30 carbon atoms, which are derived biosynthetically by the cyclization of squalene.[1] The group includes a very large number of naturally occurring members that cover an impressive variety of functional groups.[2] Many compounds of this group are reported to have interesting biological, pharmacological, or medicinal activities similar to those of retinoids and steroids, such as anti-inflammatory activity, suppression of tumor promotion, suppression of immunoglobulin synthesis, protection of the liver against toxic injury, induction of collagen synthesis, and induction of differentiation in leukemia or teratocarcinoma cells.[3] However, the potency of these triterpenoids is relatively weak. There are no systematic studies of structure−activity relationships based on chemical modification of oleanane and ursane triterpenoids.[4] We have therefore considered that bioassay-directed systematic drug design and synthesis of derivatives of oleanolic acid (**1**) and ursolic acid (**2**), which are commercially available, could be of great value in discovering novel structures with high biological potency.

The high output of nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS), which is expressed in activated macrophages, plays an important role in host defense. However, excessive production of NO also can destroy functional normal tissues during acute and chronic inflammation.[5] This phenomenon is also closely related mechanistically to carcinogenesis.[6] Thus, inhibitors of NO production in macrophages are potential anti-inflammatory and cancer chemopreventive drugs. Because oleanolic and ursolic acids are already known to have weak anti-inflammatory and anticarcinogenic activity,[3a,3b,3e,3f] we focused our attention on therapeutic agents of these diseases. For this purpose, we have adopted an assay system that measures inhibition of NO production induced by interferon-γ (IFN-γ) in mouse macrophages[7] as a preliminary screening assay system. We synthesized various oleanolic and ursolic acid derivatives and tested them as inhibitors of NO production. As a result, we have identified a series of novel olean-12-ene triterpenoids with a 1-en-3-one functionality having carboxyl, methoxycarbonyl, and nitrile groups at C-2 in ring A that show significant inhibitory activity (IC_{50} = 0.01−0.1 μM level) against production of NO induced by IFN-γ in mouse macrophages. In particular, 2-carboxy-3-oxooleana-1,12-dien-28-oic acid (**3**) had the highest activity (IC_{50} = 0.07 μM) in this group of compounds. The potency of **3** was similar to that of hydrocortisone (IC_{50} = 0.01 μM), although **3** does not act through the glucocorticoid receptor. We report here the synthesis, inhibitory activity, and structure−activity relationships of these novel triterpenoids in detail.

Chemistry

Discovery of Lead Compound. When we started this project, we had no information about a lead

† Part of this work has been reported in preliminary form: (a) Honda, T.; Finlay, H. J.; Gribble, G. W.; Suh, N.; Sporn, M. B. New enone derivatives of oleanolic acid and ursolic acid as inhibitors of nitric oxide production in mouse macrophages. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1623−1628. (b) Honda, T.; Rounds, B. V.; Bore, L.; Favaloro, F. G., Jr.; Gribble, G. W.; Suh, N.; Wang, Y.; Sporn, M. B. Novel synthetic oleanane triterpenoids: a series of highly active inhibitors of nitric oxide production in mouse macrophages. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3429−3434.

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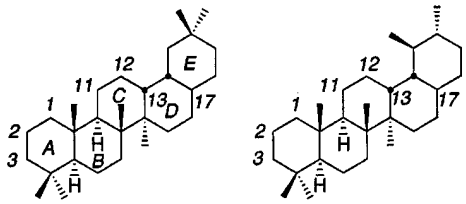
10.1021/jm000008j CCC: $19.00 © 2000 American Chemical Society
Published on Web 04/14/2000

Table 1. Preliminary Screening Results of Synthetic Oleanane and Ursane Triterpenoids

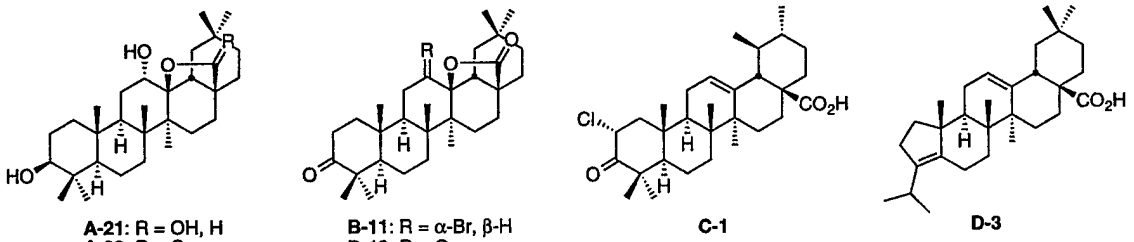


compd	skeleton	C-3	C-12	C-13	C-17	inhibition (%) at 10 μM[b]	ref
1	olean-12-ene	β-OH	H		CO_2H	38	10
2	urs-12-ene	β-OH	H		CO_2H	0	15
A-1	olean-12-ene	β-OH	H		CO_2Me	0	10
A-2	urs-12-ene	β-OH	H		CO_2Me	0	15
A-3	olean-12-ene	β-OAc	H		CO_2Me	10	10
A-4	urs-12-ene	β-OAc	H		CO_2Me	15	15
A-5	olean-12-ene	β-OAc	H		CO_2H	0	10
A-6	urs-12-ene	β-OAc	H		CO_2H	0	15
A-7	olean-12-ene	β-OH	H		CH_2OH	0	28
A-8	urs-12-ene	β-OH	H		CH_2OH	8	29
A-9	olean-12-ene	β-OAc	H		CH_2OAc	4	28
A-10	urs-12-ene	β-OAc	H		CH_2OAc	0	29
A-11	oleanane	β-OAc	α-OH	β-H	CO_2Me	0	*c*
A-12	oleanane	β-OAc	β-OH	β-H	CO_2Me	0	*c*
A-13	oleanane	β-OAc	β-OAc	β-H	CH_2OAc	0	*c*
A-14	oleanane	β-OAc	α-OH	β-H	CH_2OAc	0	*c*
A-15	oleanane	β-OH	α-OH	β-H	CH_2OH	48	*c*
A-16	oleanane	β-OH	β-OH	β-H	CH_2OH	20	*c*
A-17	oleanane	β-OH	=O	β-H	CO_2Me	0	10
A-18	oleanane	β-OAc	=O	β-H	CO_2Me	0	10
A-19	olean-12-ene	α-OH	H		CO_2H	18	30
A-20	urs-12-ene	α-OH	H		CO_2H	48	31
A-21[a]	oleanane	β-OH	α-OH	$-O-$	$-CH(OH)-$	21	32
A-22[a]	oleanane	β-OH	α-OH	$-O-$	$-CO-$	13	32
A-23	oleanane	β-OAc	12α,13α-epoxy-		CO_2Me	0	10
A-24	oleanane	β-OH	α-OH	β-OH	CH_2OH	22	32
B-1	olean-12-ene	=O	H		CO_2H	16	10
B-2	urs-12-ene	=O	H		CO_2H	22	33
B-3	olean-12-ene	=O	H		CO_2Me	24	10
B-4	urs-12-ene	=O	H		CO_2Me	16	15
B-5	olean-12-ene	=O	H		CHO	11	34
B-6	urs-12-ene	=O	H		CHO	21	34
B-7	oleana-11,13(18)-diene	=O	H		CO_2H	47	*c*
B-8	oleanane	=O	=O	β-H	CO_2Me	3	10
B-9	oleanane	=O	=O	β-H	CO_2H	37	*c*
B-10	oleanane	=O	=O	β-H	CHO	38	*c*
B-11[a]	oleanane	=O	α-Br	$-O-$	$-CO-$	4	10
B-12[a]	oleanane	=O	=O	$-O-$	$-CO-$	0	10
B-13	oleana-1,12-diene	=O	H		CO_2Me	19	9
B-14	ursa-1,12-diene	=O	H		CO_2Me	0	*d*
B-15	oleana-1,12-diene	=O	H		CO_2H	85	*d*
B-16	ursa-1,12-diene	=O	H		CO_2H	41	14
C-1[a]	urs-12-ene	=O	H		CO_2H	55	*c*
C-2	olean-12-ene	α-Cl	H		CO_2Me	2	*c*
C-3	olean-12-ene	α-Cl	H		CO_2H	0	*c*
D-1	oleana-2,12-diene	H	H		CO_2Me	3	35
D-2	oleana-2,12-diene	H	H		CO_2H	0	*c*
D-3[a]	olean-12-ene		H		CO_2H	0	*c*
E-1[a]	A-ring cleaved olean-12-ene		H		CO_2Me	21	36
E-2[a]	A-ring cleaved olean-12-ene		H		CO_2H	33	37
E-3[a]	A-ring cleaved urs-12-ene		H		CO_2H	39	37
E-4[a]	A-ring cleaved olean-12-ene		H		CO_2H	22	37
E-5[a]	A-ring cleaved urs-12-ene		H		CO_2H	55	37
E-6[a]	A-ring cleaved urs-12-ene		H		CO_2H	10	37
F-1[a]	C-ring cleaved oleanane	β-OAc			CH_2OAc	52	*c*
F-2[a]	C-ring cleaved oleanane	β-OAc			CH_2OAc	12	*c*
F-3[a]	C-ring cleaved oleanane	β-OAc			CH_2OAc	52	*c*
F-4[a]	C-ring cleaved oleanane	=O			CO_2H	28	*c*

Table 1 (Continued)

compd	skeleton	C-3	C-12	C-13	C-17	inhibition (%) at 10 μM[b]	ref
G-1[a]	olean-12-ene		H		CO_2Me	0	36
G-2[a]	olean-12-ene		H		CO_2H	51	37
hydrocortisone						80	

[a] Structure shown below this table. [b] Details of the evaluation method are described in the Experimental Section. [c] Unknown compound (synthesis and spectral data will be published elsewhere). [d] Unknown compound (synthesis and spectral data are shown in this paper).

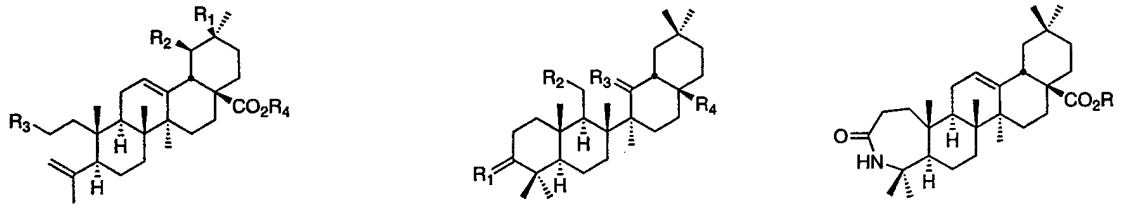


A-21: R = OH, H
A-22: R = O

B-11: R = α-Br, β-H
B-12: R = O

C-1

D-3

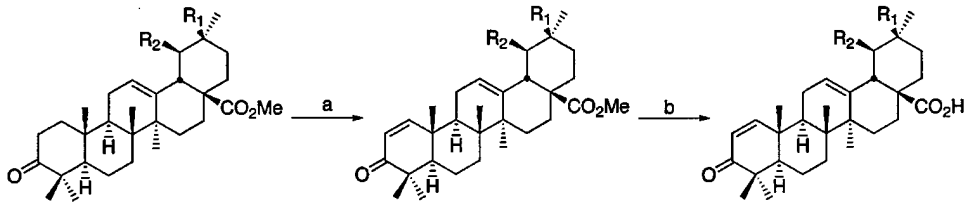


E-1: $R_1 = R_4 = Me$, $R_2 = H$, $R_3 = CN$
E-2: $R_1 = Me$, $R_2 = R_4 = H$, $R_3 = CN$
E-3: $R_1 = R_4 = H$, $R_2 = Me$, $R_3 = CN$
E-4: $R_1 = Me$, $R_2 = R_4 = H$, $R_3 = CHO$
E-5: $R_1 = R_4 = H$, $R_2 = Me$, $R_3 = CHO$
E-6: $R_1 = R_4 = H$, $R_2 = Me$, $R_3 = CO_2H$

F-1: R_1 = β-OAc, $R_2 = CHO$, $R_3 = O$, $R_4 = CH_2OAc$
F-2: R_1 = β-OAc, $R_2 = CH_2OH$, $R_3 = OH$, $R_4 = CH_2OAc$
F-3: R_1 = β-OAc, $R_2 = CO_2H$, $R_3 = O$, $R_4 = CH_2OAc$
F-4: $R_1 = R_3 = O$, $R_2 = CO_2H$, $R_4 = CO_2H$

G-1: R = Me
G-2: R = H

Scheme 1[a]



B-3: $R_1 = Me$, $R_2 = H$
B-4: $R_1 = H$, $R_2 = Me$

B-13: $R_1 = Me$, $R_2 = H$
B-14: $R_1 = H$, $R_2 = Me$

B-15: $R_1 = Me$, $R_2 = H$
B-16: $R_1 = H$, $R_2 = Me$

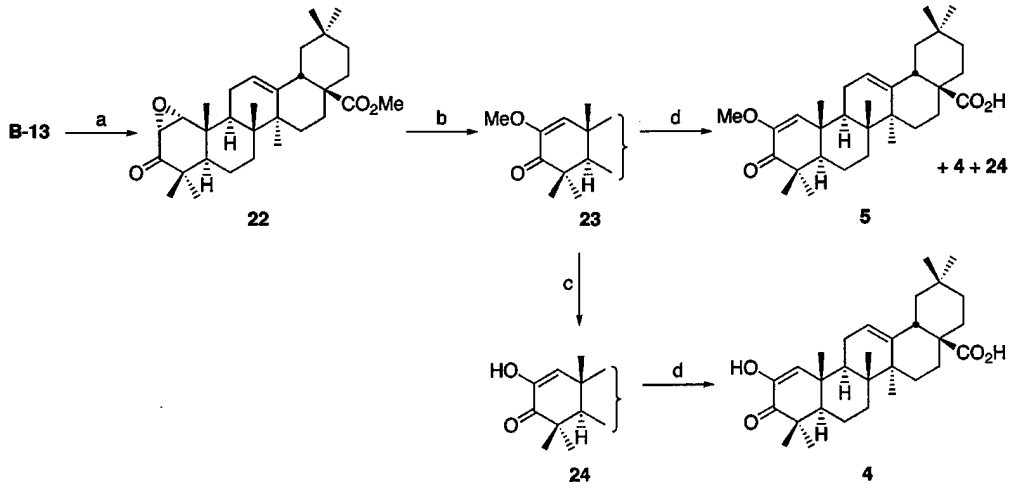
[a] Reagents: (a) PhSeCl, EtOAc; *m*CPBA, pyr, EtOAc; (b) LiI, DMF.

compound. Therefore, about 60 oleanolic and ursolic acid derivatives were initially randomly synthesized. They are divided into seven categories: 3-hydroxy derivatives, **A**; 3-oxo derivatives, **B**; chloro derivatives, **C**; dehydroxyoleanane derivatives, **D**; A-ring cleaved derivatives, **E**; C-ring cleaved oleanane derivatives, **F**; and lactams, **G** (see Table 1). In the preliminary screen of these derivatives for inhibition of production of NO induced by IFN-γ in mouse macrophages, 3-oxooleana-1,12-dien-28-oic acid (**B-15**) was found to show significant activity (inhibition: 85% at 10 μM, $IC_{50} = 5.6$ μM). (See Tables 1 and 2.)

Design and Synthesis of New Derivatives. When **B-15** is compared with the other derivatives, it has the following features: first, it is an oleanane; second, it has a 1-en-3-one functionality in ring A; third, it has a carboxyl group at C-17. We focused our attention on the 1-en-3-one functionality in ring A among these features. We therefore designed novel olean- and urs-12-ene triterpenoids with a 1-en-3-one functionality having a substituent at C-2 in ring A, **3**–**19**, and novel triterpenoid–steroid hybrid compounds, **20** and **21**[8] (see Table 2). The syntheses of these newly designed derivatives and compounds **B-13**–**B-16** are illustrated in Schemes 1–6.

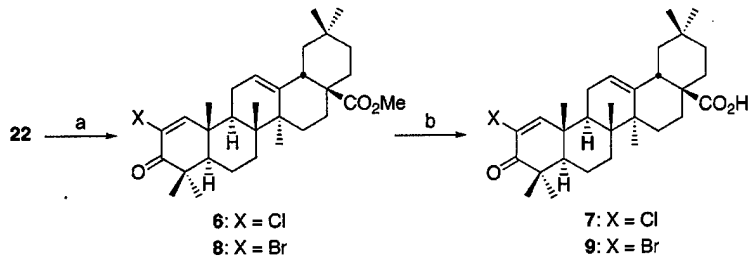
Ester **B-13**[9] was synthesized in 62% yield by introduction of a double bond at C-1 of methyl oleanonate (**B-3**)[10] with phenylselenenyl chloride (PhSeCl) in ethyl acetate and sequential addition of pyridine and *m*-chloroperbenzoic acid.[11,12] Acid **B-15** was synthesized in 85% yield by halogenolysis of **B-13** with lithium iodide in *N*,*N*-dimethylformamide (DMF).[13] Similarly, acid **B-16**[14] was synthesized in 58% yield via ester **B-14** from methyl ursonate (**B-4**).[15] Epoxide **22**[9] was prepared in 99% yield by epoxidation of **B-13** with alkaline hydrogen peroxide. Treatment of **22** with sodium methoxide[16] gave enone **23** (yield, 87%; 98% based on recovered **22**). Diosphenol **24** was synthesized by demethylation of the methyl enol ether at C-2 of **23** with hydrochloric acid in acetic acid (yield, 81%). Halogenolysis of **24** gave acid **4** (yield, 18%). Halogenolysis of **23** gave a desired partial demethylated product **5** in 28% (41% based on recovered

Scheme 2[a]



[a] Reagents: (a) 30% H_2O_2, NaOH(aq), THF; (b) NaOMe, MeOH; (c) HCl, AcOH; (d) LiI, DMF.

Scheme 3[a]



[a] Reagents: (a) HX, AcOH, $CHCl_3$; (b) LiI, DMF.

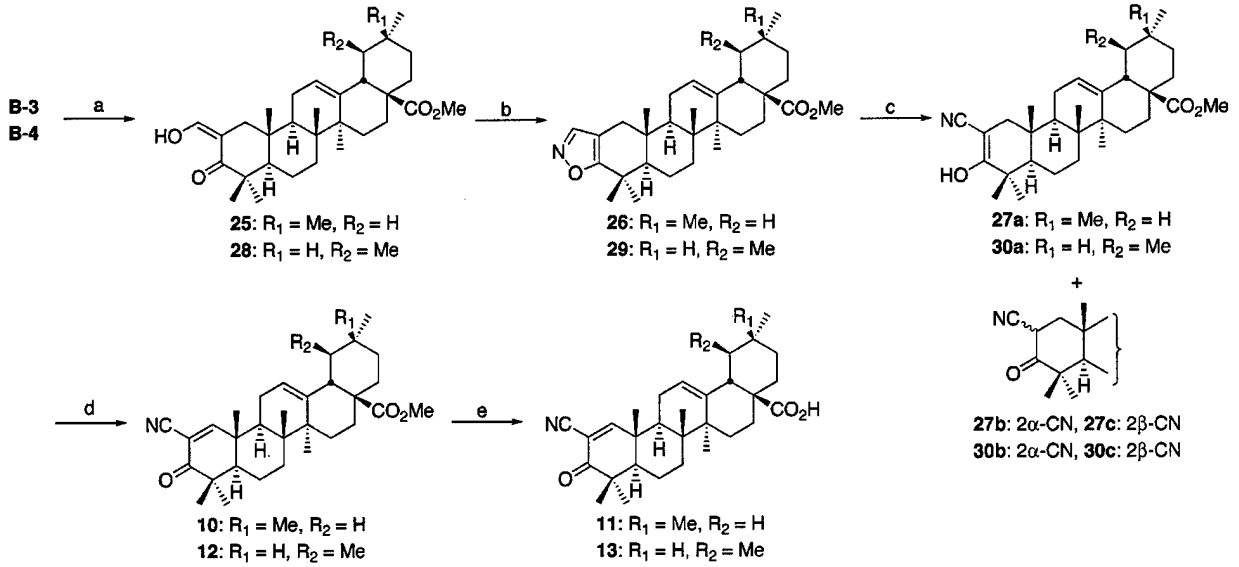
23) yield.[17] Chloride **6** was synthesized in 81% yield from **22** with hydrogen chloride in acetic acid and chloroform.[18] Halogenolysis of **6** gave chloride **7** in 77% yield. Similarly, bromides **8** and **9** were prepared from **22** and **8** (yield, 96% and 76%), respectively. Hydroxymethylene **25**[19,20] was prepared in 95% yield by formylation of **B-3** with ethyl formate in the presence of sodium methoxide in benzene.[21] Isoxazole **26** was prepared in 86% yield by condensation of **25** with hydroxylamine.[22] Cleavage of the isoxazole moiety of **26** with sodium methoxide gave nitrile **27** in 99% yield.[22] ^{1}H NMR showed that **27** is a mixture of three tautomers [**27a**, **27b** (2α-cyano), and **27c** (2β-cyano)] and that **27a** is the major one in $CDCl_3$. Enone **10** was prepared in 88% yield by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation of **27** in benzene, although the same method as for **B-13** gave **10** in only 35% yield. Halogenolysis of **10** gave acid **11** in 71% (91% based on recovered **10**) yield. Similarly, ursane derivative **12** was synthesized in 52% yield via **28**,[20,23] **29**, and **30** from **B-4**. Acid **13** was prepared in 74% yield by halogenolysis of **12**. Enal **14** was prepared from **25** by PhSeCl–pyridine in methylene chloride and sequential addition of 30% hydrogen peroxide[24] (yield, 71%; 79% based on recovered **25**). Halogenolysis of **14** did not give acid **15** but a complex mixture. Therefore, the synthesis of acid **15** from oleanonic acid (**B-1**)[10] was attempted. Formylation of **B-1** with ethyl formate in the presence of sodium methoxide in tetrahydrofuran gave **32**[20] (yield, 45%; 66% based on recovered **B-1**). Acid **15** was prepared from **32** according to the same method as for **14** (yield, 71%; 84% based on recovered **32**). Jones oxidation of **14** gave acid **16** in 30% (39% based on recovered **14**) yield. Because this yield was not enough to synthesize derivatives **3** and **17**–**19** from **16**, an alternative route was adopted. Ester **31** was prepared in 74% (89% based on recovered **B-3**) yield from **B-3** by Stiles' reagent (methoxymagnesium methyl carbonate) in DMF,[25] followed by methylation with diazomethane. ^{1}H NMR showed that **31** is the single tautomer in $CDCl_3$ as depicted in Scheme 5. Enone **17** was prepared from **31** according to the same method as for **14** (yield, 83%; 90% based on recovered **31**). Hydrolysis of **17** with potassium hydroxide in aqueous methanol gave acid **16** selectively in 97% yield because the methoxycarbonyl group at C-17 of **17** is sterically hindered. Halogenolysis of **16** gave dicarboxylic acid **3** in 58% yield. Methylation of **3** with methanol under acidic conditions gave ester **18** selectively in 78% yield because of the steric hindrance of the carboxylic acid at C-17 of **3**. Amide **19** was prepared selectively in 96% yield from **17** with saturated ammonia–methanol.

Biological Results and Discussion

The inhibitory activities [IC_{50} (μM) value] of compounds **B-1**, **B-13**, **B-15**, **B-16**, **1**–**21**, and hydrocortisone (a positive control) on NO production induced by IFN-γ in mouse macrophages are shown in Table 2. These derivatives are arranged according to the strength of Taft's σ^* values[26] of substituents at C-2. These results provide the following interesting structure–activity relationships:

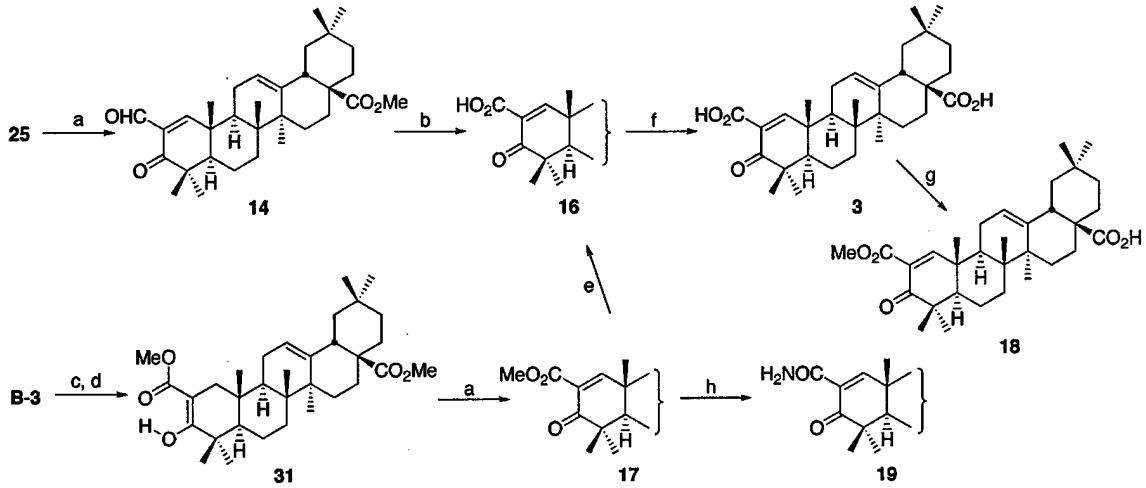
(1) In the A ring, a 1-en-3-one functionality is important for significant activity. The lead compound **B-15** is much more potent than the C-3 ketone **B-1** and the

Scheme 4[a]



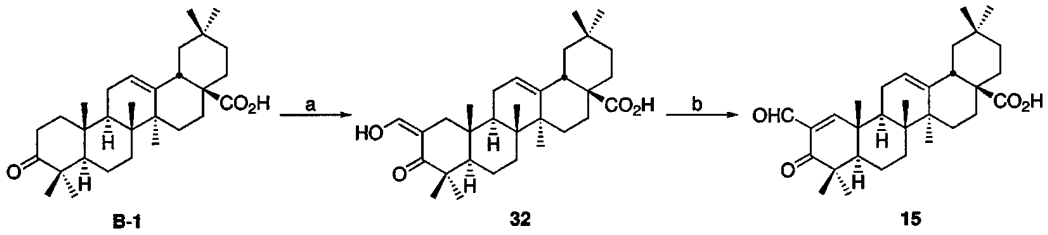
[a] Reagents: (a) HCO_2Et, NaOMe, PhH; (b) $NH_2OH{\cdot}HCl$, aq EtOH; (c) NaOMe, Et_2O, MeOH; (d) DDQ, PhH; (e) LiI, DMF.

Scheme 5[a]



[a] Reagents: (a) PhSeCl, pyr, CH_2Cl_2; 30% H_2O_2, CH_2Cl_2; (b) Jones; (c) Stiles' reagent, DMF; (d) CH_2N_2, Et_2O, THF; (e) KOH, aq MeOH; (f) LiI, DMF; (g) H_2SO_4, MeOH; (h) NH_3, MeOH.

Scheme 6[a]



[a] Reagents: (a) HCO_2Et, NaOMe, THF; (b) PhSeCl, pyr, CH_2Cl_2; 30% H_2O_2, CH_2Cl_2.

C-3 alcohol **1** (oleanolic acid). Also, the ursane derivative **B-16** is more potent than the C-3 alcohol **2** (ursolic acid).

(2) A correlation between Taft's σ^* values of substituents at C-2 and biological activity is not observed. This result shows that the activity does not depend on the strength of electron-withdrawing effect of a substituent at C-2.

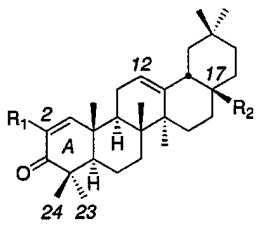
(3) Carboxyl, methoxycarbonyl, and nitrile groups at C-2 enhance activity. Compounds **3**, **10**, **11**, **16**, and **17** are about 10−100 times more potent than **B-15**. In particular, **3** showed the highest activity (IC_{50} = 0.07 μM) in this series of compounds. The potency of **3** was similar to that of hydrocortisone (IC_{50} = 0.01 μM).

(4) Hydroxyl, aminocarbonyl, methoxy, chloride, and bromide groups decrease activity. Compounds **4**−**9** and **19** are much less potent than **B-15**.

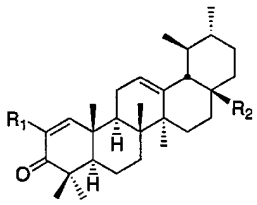
(5) A formyl group does not confer activity but only toxicity.

(6) 23,24-Dimethyl groups are important for signifi-

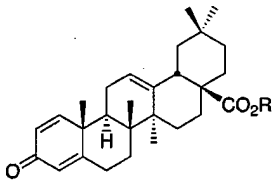
Table 2. Activity of Olean- and Urs-12-ene Triterpenoids with Various 1-En-3-one Functionalities



3-oxooleana-1,12-diene



3-oxoursa-1,12-diene



20: R = Me
21: R = H

compd	skeleton[a]	R_1 at C-2	R_2 at C-17	Taft's σ^* value of R_1	formula	analyses[b]	activity[c] IC_{50} (μM)
B-13	O	H	CO_2Me		$C_{31}H_{46}O_3$	ref 9	31
B-15	O	H	CO_2H		$C_{30}H_{44}O_3 \cdot 3/4H_2O$	C, H	5.6
20	D	H	CO_2Me		$C_{29}H_{40}O_3 \cdot 1/4H_2O$	C, H	>40
21	D	H	CO_2H		$C_{28}H_{38}O_3 \cdot 1/3H_2O$	C, H	13
B-16	U	H	CO_2H		$C_{30}H_{44}O_3$	ref 14	13
5	O	OH	CO_2H	1.34	$C_{30}H_{44}O_4 \cdot 1/2H_2O$	C, H	27
19	O	$CONH_2$	CO_2Me	1.68	$C_{32}H_{47}O_4N \cdot 3/4H_2O$	C, H, N	14
4	O	OMe	CO_2H	1.81	$C_{31}H_{46}O_4 \cdot 1/2H_2O$	C, H	30
17	O	CO_2Me	CO_2Me	2.00	$C_{33}H_{48}O_5$	C, H	0.9
18	O	CO_2Me	CO_2H	2.00	$C_{32}H_{46}O_5$	C, H	2.2
16	O	CO_2H	CO_2Me	2.08	$C_{32}H_{46}O_5 \cdot 1/2H_2O$	C, H	0.8
3	O	CO_2H	CO_2H	2.08	$C_{31}H_{44}O_5$	C, H	0.07
14	O	CHO	CO_2Me	2.15	$C_{32}H_{46}O_4$	C, H	toxic[d]
15	O	CHO	CO_2H	2.15	$C_{31}H_{44}O_4 \cdot 1/2H_2O$	C, H	toxic[d]
8	O	Br	CO_2Me	2.84	$C_{31}H_{45}O_3Br$	C, H	>40
9	O	Br	CO_2H	2.84	$C_{30}H_{43}O_3Br \cdot H_2O$	C, H	7.3
6	O	Cl	CO_2Me	2.96	$C_{31}H_{45}O_3Cl$	C, H	>40
7	O	Cl	CO_2H	2.96	$C_{30}H_{43}O_3Cl \cdot 1/4H_2O$	C, H	>40
10	O	CN	CO_2Me	3.30	$C_{32}H_{45}O_3N \cdot 1/4H_2O$	C, H, N	0.7
11	O	CN	CO_2H	3.30	$C_{31}H_{43}O_3N \cdot 1/2H_2O$	C, H, N	0.6
12	U	CN	CO_2Me	3.30	$C_{32}H_{45}O_3N \cdot 3/4H_2O$	C, H, N	5.1
13	U	CN	CO_2H	3.30	$C_{31}H_{43}O_3N \cdot H_2O$	C, H, N	6.2
B-1		oleanonic acid			$C_{30}H_{46}O_3$	ref 10	37
1		oleanolic acid			$C_{30}H_{48}O_3$	ref 10	>40
2		ursolic acid			$C_{30}H_{48}O_3$	ref 15	toxic[e]
		hydrocortisone					0.01

[a] O, 3-oxooleana-1,12-diene; D, 23,24-dinor-3-oxooleana-1,4,12-triene; U, 3-oxoursa-1,12-diene. [b] C, H, and N analyses were within ±0.4% of the theoretical values. [c] Details of the evaluation method are described in the Experimental Section. IC_{50} values of **3** and hydrocortisone were determined in the range of 0.1 pM−1 μM (10-fold dilutions). The other compounds were assayed in the range of 0.01−40 μM (4-fold dilutions). Values are an average of two separate experiments. [d] Compounds **14** and **15** were toxic to cells above 1 μM and were not active below 1 μM. [e] Ursolic acid (**2**) was toxic to cells above 10 μM and was not active below 10 μM.

cant activity. **B-15** is more potent than 23,24-dinor-olean-1-en-3-one derivative **21**.

(7) The oleanane skeleton is more potent than the ursane skeleton. **B-15**, **10**, and **11** are more potent than **B-16**, **12**, and **13**, respectively.

(8) The role of methoxycarbonyl and carboxyl groups at C-17 is ambiguous. In some analogues, the carboxyl group is more potent than the methoxycarbonyl group: acids **B-15**, **3**, **9**, and **21** are more potent than esters **B-13**, **16**, **8**, and **20**, respectively. For other analogues, the carboxyl and methoxycarbonyl groups show similar activity: acids **11** and **13** show similar activity to esters **10** and **12**, respectively. Lastly, acid **18** is less potent than ester **17**.

The inhibitory activity of new triterpenoids **3** and **11** was not blocked by the glucocorticoid antagonist, RU-486,[27] which reverses the action of hydrocortisone (see Figure 1). These data strongly suggest that the actions of these triterpenoids on the iNOS system are not mediated by their interaction with the glucocorticoid receptor.

On the basis of these structure−activity relationships, further lead optimization is in progress. Further biological evaluation and studies on the mechanism of action of **3** are also in progress.

Experimental Section

General Experimental Procedures. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Jasco DIP-181 digital polarimeter. UV and IR spectra were recorded on a Hewlett-Packard 8451A UV/VIS spectrophotometer and a Perkin-Elmer 600 series FTIR spectrophotometer, respectively. ^1H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Varian XL-300 Fourier transform spectrometer. The chemical shifts are reported in δ (ppm) using the δ 7.27 signal of $CHCl_3$ (^1H NMR) and the δ 77.23 signal of $CDCl_3$ (^{13}C NMR) as internal standards. Low-resolution mass spectra and high-resolution MS data were obtained on a Micromass 70-VSE unless otherwise stated. Elemental microanalysis was performed by Atlantic Microlab Inc. TLC and preparative TLC (prep-TLC) were performed with Merck precoated TLC plates silica gel 60 F_{254}. Flash column chromatography was done with Select Scientific silica gel (230−400 mesh). The standard work up method was as follows: an organic extract was washed with saturated aqueous $NaHCO_3$ solution (three times) followed by saturated aqueous NaCl solution (three times), then dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated in vacuo.

Methyl 3-Oxooleana-1,12-dien-28-oate (B-13).[9] A solution of methyl oleanonate (**B-3**)[10] (2.00 g, 4.27 mmol) and phenylselenenyl chloride (98%) (1.00 g, 5.12 mmol) in EtOAc (85 mL) was stirred at room temperature for 3 h. To the stirred

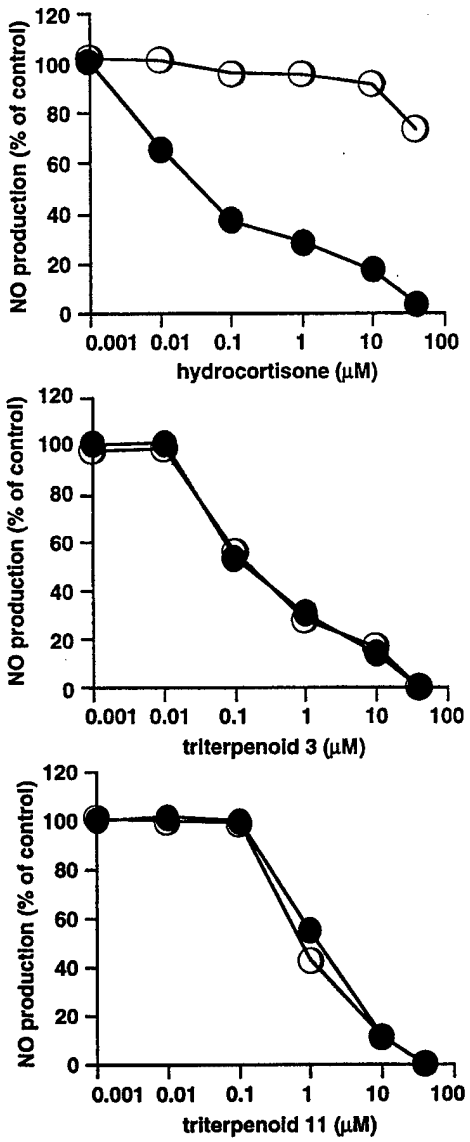


Figure 1. Blockage by glucocorticoid antagonist RU486 of hydrocortisone-inhibited NO production but not of triterpenoid (**3** and **11**) inhibited NO production in primary mouse macrophages. Macrophage cells were incubated with IFN-γ (20 ng/mL) together with hydrocortisone or triterpenoids without RU486 (●); in some cases RU486 (1 μM) was added simultaneously to both hydrocortisone- and triterpenoid-treated cell wells (○). RU486 itself does not interfere with NO production at the concentration tested.

mixture, saturated aqueous $NaHCO_3$ solution was added. After most of the aqueous layer was removed, pyridine (844 mg, 10.7 mmol) and *m*-chloroperbenzoic acid (50–60%) (3.68 g, 10.7 mmol) were added to the organic layer. The mixture was stirred at room temperature for 1 h. The mixture was washed with 5% aqueous NaOH solution (three times), saturated aqueous NH_4Cl solution (three times), and saturated aqueous NaCl solution (three times); dried over anhydrous $MgSO_4$; and filtered. The filtrate was evaporated in vacuo to give a solid. The solid was subjected to flash column chromatography [hexanes–EtOAc (5:1)] to give **B-13** as a crystalline solid (1.23 g, 62%): mp 159–161 °C; $[\alpha]^{25}_D$ +103° (*c* 0.64, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 230 (3.92) nm. IR (KBr): 2946, 2867, 1728, 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.04 (1H, d, J = 10.1 Hz), 5.81 (1H, d, J = 10.1 Hz), 5.36 (1H, t, J = 3.7 Hz), 3.64, (3H, s), 2.90 (1H, dd, J = 4.6, 13.9 Hz), 1.17 (3H, s), 1.16 (6H, s), 1.10, 0.94, 0.91, 0.83 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 205.5, 178.4, 159.3, 144.5, 125.2, 121.9, 53.6, 51.8, 47.0, 45.9, 44.7, 42.2, 42.0, 41.7, 40.3, 39.7, 34.1, 33.3, 32.7, 32.5, 30.9, 28.0, 27.9, 26.0, 23.8, 23.5, 23.2, 21.8, 19.1, 18.8, 17.5. EIMS (70 eV) *m/z*: 466 $[M]^+$ (73), 451 (11), 407 (31), 262 (57), 203 (100). HREIMS: Calcd for $C_{31}H_{46}O_3$: 466.3447. Found: 466.3446.

Methyl 3-Oxoursa-1,12-dien-28-oate (B-14). **B-14** was prepared from methyl ursonate (**B-4**)[15] according to the same method as for **B-13** to give an amorphous solid (66%): $[\alpha]^{26}_D$ +93° (*c* 0.77, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 232 (3.95) nm. IR (KBr): 2974, 2935, 2871, 1725, 1669 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.06 (1H, d, J = 10.1 Hz), 5.81 (1H, d, J = 10.1 Hz), 5.33 (1H, t, J = 3.8 Hz), 3.63 (3H, s), 2.28 (1H, d, J = 11.5 Hz), 1.17, 1.15 (each 3H, s), 1.10 (6H, s), 0.95 (3H, d, J = 5.4 Hz), 0.87 (3H, d, J = 6.3 Hz), 0.85 (3H, s). ^{13}C NMR ($CDCl_3$): δ 205.5, 178.2, 159.5, 139.0, 125.2, 125.0, 53.7, 53.3, 51.7, 48.4, 44.7, 42.6, 41.9, 40.5, 39.5, 39.2, 39.1, 36.8, 33.0, 30.8, 28.2, 28.1, 24.4, 23.7, 23.5, 21.8, 21.4, 19.1, 19.0, 17.7, 17.2. EIMS (70 eV) *m/z*: 466 $[M]^+$ (14), 406 (12), 262 (74), 203 (100). HREIMS: Calcd for $C_{31}H_{46}O_3$: 466.3447. Found: 466.3442.

3-Oxooleana-1,12-dien-28-oic Acid (B-15). A mixture of **B-13** (100 mg, 0.21 mmol) and LiI (500 mg) in dry DMF (2 mL) was heated under reflux for 6 h. The mixture was acidified with 5% aqueous HCl solution and then extracted with a mixture of CH_2Cl_2 and Et_2O (1:2) three times. The extract was worked up according to the standard method to give a solid (110 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (5:1) followed by hexanes–EtOAc (2:1)] to give **B-15** as an amorphous solid (82 mg, 85%): $[\alpha]^{26}_D$ +103° (*c* 0.45, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 230 (3.75) nm. IR (KBr): 2941, 2866, 1732, 1695, 1671 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.04 (1H, d, J = 10.2 Hz), 5.81 (1H, d, J = 10.2 Hz), 5.35 (1H, t, J = 3.3 Hz), 2.86 (1H, dd, J = 4.2, 13.4 Hz), 1.16, 1.152, 1.147, 1.07, 0.94, 0.91, 0.84 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 205.5, 184.5, 159.2, 144.2, 125.3, 122.1, 53.5, 46.8, 45.8, 44.7, 42.1, 41.9, 41.3, 40.2, 39.7, 34.0, 33.3, 32.6, 32.5, 30.9, 28.0, 27.8, 26.0, 23.7, 23.5, 23.0, 21.8, 19.0, 18.9, 17.7. EIMS (70 eV) *m/z*: 452 $[M]^+$ (8.8), 437 (3.8), 406 (6.8), 248 (80), 233 (14), 203 (100). HREIMS: Calcd for $C_{30}H_{44}O_3$: 452.3290. Found: 452.3289. Anal. (Table 2).

3-Oxoursa-1,12-dien-28-oic Acid (B-16).[14] **B-16** was prepared from **B-14** according to the same method as for **B-15** to give an amorphous solid (88%): $[\alpha]^{26}_D$ +91° (*c* 0.84, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 230 (3.99) nm. IR (KBr): 3306, 2973, 2930, 2870, 1729, 1695, 1669 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.07 (1H, d, J = 10.1 Hz), 5.82 (1H, d, J = 10.1 Hz), 5.33 (1H, t, J = 3.7 Hz), 2.24 (1H, d, J = 11.2 Hz), 1.18, 1.16, 1.11, 1.09 (each 3H, s), 0.96 (3H, d, J = 6.1 Hz), 0.88 (3H, d, J = 6.4 Hz), 0.88 (3H, s). ^{13}C NMR ($CDCl_3$): δ 205.5, 183.9, 159.4, 138.8, 125.3, 53.6, 52.9, 48.3, 44.7, 42.5, 41.9, 40.5, 39.6, 39.2, 39.0, 36.8, 32.9, 30.8, 28.2, 28.1, 24.2, 23.7, 23.4, 21.8, 21.3, 19.0, 17.8, 17.2. FABMS (NBA) *m/z*: 453 $[M + H]^+$ (100) (by a Micromass ZAB-SE). HRFABMS: Calcd for $C_{30}H_{44}O_3$ + H: 453.3369. Found: 453.3335 (by a Micromass 70-SE-4F).

2-Carboxy-3-oxooleana-1,12-dien-28-oic Acid (3). A mixture of **16** (109 mg, 0.21 mmol) and LiI (520 mg) in dry DMF (1.5 mL) was heated under reflux for 1 h. After 5% aqueous HCl solution was added, the acidic mixture was extracted with EtOAc three times. The extract was washed with water (three times) and saturated aqueous NaCl solution (three times), dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated in vacuo to give a residue (108 mg). The residue was subjected to flash column chromatography [CH_2Cl_2–MeOH (15:1) followed by CH_2Cl_2–MeOH (10:1)] to afford **3** as a crystalline solid (61 mg, 58%): mp >250 °C dec; $[\alpha]^{26}_D$ +81° (*c* 0.53, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 234 (3.88) nm. IR (KBr): 3389, 2943, 2872, 1752, 1696, 1637 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.43 (1H, s), 5.37 (1H, t, J = 3.5 Hz), 2.87 (1H, dd, J = 3.8, 13.9 Hz), 1.25, 1.22, 1.18, 1.15, 0.95, 0.93, 0.88 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 209.0, 183.9, 173.2, 165.2, 144.2, 123.4, 121.7, 52.4, 46.8, 45.7, 45.5, 42.3, 41.4, 41.1, 40.6, 40.4, 34.0, 33.2, 32.5, 32.3, 30.9, 28.4, 27.8, 26.0, 23.7, 23.5, 23.0, 22.0, 19.0, 18.4, 17.8. EIMS (70 eV) *m/z*: 496 $[M]^+$ (3.0), 478 (3.4), 452 (7.6), 248 (56), 231 (35), 203 (100). HREIMS: Calcd for $C_{31}H_{44}O_5$: 496.3189. Found: 496.3196. Anal. (Table 2).

2-Hydroxy-3-oxooleana-1,12-dien-28-oic Acid (4). **4** was prepared from **24** according to the same method as for **B-15**

except that the reaction time was 2 h. The reaction mixture was subjected to flash column chromatography [hexanes–EtOAc (5:1) followed by hexanes–EtOAc (4:1)] to give **4** as an amorphous solid (18%): $[\alpha]^{25}_D$ +99° (*c* 0.46, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 272 (3.71) nm. IR (KBr): 3434, 2938, 1698, 1667, 1649 cm^{-1}. ^1H NMR ($CDCl_3$): δ 6.35 (1H, s), 5.96 (1H, brs), 5.34 (1H, t, J = 3.5 Hz), 2.86 (1H, dd, J = 3.8, 13.9 Hz), 1.23, 1.22, 1.14, 1.11, 0.94, 0.92, 0.83 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 201.2, 184.0, 144.1, 143.9, 128.4, 122.3, 54.0, 46.8, 45.8, 44.1, 43.3, 42.2, 41.3, 40.2, 38.7, 34.0, 33.3, 32.6, 30.9, 27.8, 27.4, 26.1, 23.8, 23.6, 23.0, 22.0, 19.9, 18.9, 17.7. EIMS (70 eV) *m/z*: 468 $[M]^+$ (3.2), 248 (13), 203 (23), 149 (42), 84 (100). HREIMS: Calcd for $C_{30}H_{44}O_4$: 468.3240. Found: 468.3222. Anal. (Table 2).

2-Methoxy-3-oxooleana-1,12-dien-28-oic Acid (5). A mixture of **23** (230 mg, 0.46 mmol) and LiI (1045 mg) in dry DMF (3.5 mL) was heated under reflux for 4 h. The reaction mixture was worked up according to the same method as for **B-15** to give a solid (230 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (3:1) followed by hexanes–EtOAc (2:1), then hexanes–EtOAc (1:1)] to give **24** (35 mg; 16%, 23% based on recovered **23**), **23** (74 mg), **4** (27 mg; 12%, 18% based on recovered **23**), and **5** as an amorphous solid (63 mg; 28%, 41% based on recovered **23**): $[\alpha]^{26}_D$ +96° (*c* 0.29, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 266 (3.84) nm. IR (KBr): 3307, 2947, 2862, 1732, 1693, 1622 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.96 (1H, s), 5.36 (1H, t, J = 3.3 Hz), 3.56 (3H, s), 2.87 (1H, dd, J = 4.2, 13.9 Hz), 1.17 (9H, s), 1.11, 0.94, 0.91, 0.84 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 200.0, 184.4, 149.1, 144.4, 126.1, 122.1, 55.0, 53.2, 46.8, 45.9, 45.4, 43.3, 42.2, 41.3, 40.2, 38.5, 34.0, 33.3, 32.5, 30.9, 28.6, 27.8, 26.1, 23.8, 23.0, 22.0, 20.4, 19.2, 17.6. EIMS (70 eV) *m/z*: 482 $[M]^+$ (11), 415 (6.5), 245 (18), 203 (33), 157 (100). HREIMS: Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3375. Anal. (Table 2).

Methyl 2-Chloro-3-oxooleana-1,12-dien-28-oate (6). A solution of **22** (99 mg, 0.21 mmol) in AcOH including 1 M HCl (2.5 mL) and $CHCl_3$ (2.5 mL) was stirred at room temperature overnight. The mixture was diluted with CH_2Cl_2. After it was washed with water three times, it was worked up according to the standard method to give a solid (96 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (6:1)] to afford **6** as an amorphous solid (84 mg, 81%): $[\alpha]^{26}_D$ +98° (*c* 0.26, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 250 (3.91) nm. IR (KBr): 2943, 2866, 1727, 1689 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.22 (1H, s), 5.34 (1H, t, J = 3.5 Hz), 3.62 (3H, s), 2.89 (1H, dd, J = 4.2, 13.7 Hz), 1.203, 1.197 (each 3H, s), 1.14 (6H, s), 0.93, 0.90, 0.80 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 197.4, 178.3, 155.0, 144.5, 129.8, 121.5, 53.3, 51.8, 46.9, 46.3, 45.8, 42.2, 42.1, 41.64, 41.57, 40.3, 34.0, 33.3, 32.4, 30.9, 28.4, 27.8, 26.0, 23.8, 23.5, 23.1, 22.1, 19.1, 18.8, 17.5. EIMS (70 eV) *m/z*: 500 $[M]^+$ (21), 262 (27), 247 (96), 203 (100). HREIMS: Calcd for $C_{31}H_{45}O_3Cl$: 500.3057. Found: 500.3060. Anal. (Table 2).

2-Chloro-3-oxooleana-1,12-dien-28-oic Acid (7). **7** was prepared from **6** according to the same method as for **B-15** except that the reaction time was 4 h. The reaction mixture was subjected to flash column chromatography [hexanes–EtOAc (4:1) followed by hexanes–EtOAc (3:1)] to give **7** as an amorphous solid (77%): $[\alpha]^{26}_D$ +88° (*c* 0.50, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 252 (3.20) nm. IR (KBr): 3297, 2943, 2870, 1733, 1691, 1601 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.23 (1H, s), 5.35 (1H, t, J = 3.3 Hz), 2.86 (1H, dd, J = 4.3, 13.8 Hz), 1.22, 1.21, 1.16, 1.13, 0.94, 0.92, 0.84 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 197,4, 184.4, 154.9, 144.3, 129.9, 121.8, 53.2, 46.8, 46.4, 45.8, 42.21, 42.16, 41.6, 41.3, 40.3, 34.0, 33.3, 32.5, 32.4, 30.9, 28.5, 27.8, 26.0, 23.7, 23.5, 23.0, 22.1, 19.0, 18.9, 17.7. EIMS (70 eV) *m/z*: 486 $[M]^+$ (25), 248 (100), 203 (96). HREIMS: Calcd for $C_{30}H_{43}O_3Cl$: 486.2901. Found: 486.2898. Anal. (Table 2).

Methyl 2-Bromo-3-oxooleana-1,12-dien-28-oate (8). A solution of **22** (220 mg, 0.46 mmol) in AcOH including 1 M HBr (4.9 mL) and $CHCl_3$ (6.1 mL) was stirred at room temperature for 1 h. The mixture was diluted with CH_2Cl_2. After it was washed with water three times, it was worked up according to the standard method to give a solid (260 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (6:1)] to afford **8** as an amorphous solid (238 mg, 96%): $[\alpha]^{26}_D$ +88° (*c* 0.51, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 260 (3.69) nm. IR (KBr): 2943, 2870, 1733, 1691, 1601 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.49 (1H, s), 5.35 (1H, t, J = 3.5 Hz), 3.63 (3H, s), 2.90 (1H, dd, J = 4.0, 13.8 Hz), 1.20, 1.15 (each 6H, s), 0.94, 0.91, 0.81 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 197.3, 178.3, 159.5, 144.6, 121.8, 121.5, 53.3, 51.8, 46.9, 46.5, 45.8, 43.1, 42.3, 42.1, 41.7, 40.3, 34.0, 33.3, 32.4, 30.9, 28.7, 27.8, 26.0, 23.8, 23.6, 23.2, 22.3, 19.1, 18.7, 17.5. EIMS (70 eV) *m/z*: 546 (5.0) and 544 (5.2) $[M]^+$, 262 (8.5), 203 (24), 118 (100), 116 (100). HREIMS: Calcd for $C_{31}H_{45}O_3Br$: 544.2552. Found: 544.2553. Anal. (Table 2).

2-Bromo-3-oxooleana-1,12-dien-28-oic Acid (9). **9** was prepared from **8** according to the same method as for **B-15** except that the reaction time was 4 h. The reaction mixture was subjected to flash column chromatography [hexanes–EtOAc (4:1) followed by hexanes–EtOAc (3:1)] to give **9** as an amorphous solid (76%): $[\alpha]^{26}_D$ +82° (*c* 0.31, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 260 (3.52) nm. IR (KBr): 3434, 2939, 2870, 1727, 1686, 1601 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.49 (1H, s), 5.35 (1H, t, J = 3.4 Hz), 2.86 (1H, dd, J = 4.2, 13.7 Hz), 1.21 (6H, s), 1.16, 1.14, 0.94, 0.92, 0.83 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 197.2, 184.4, 159.3, 144.3, 121.84, 121.79, 53.3, 46.8, 46.5, 45.8, 43.1, 42.2, 42.0, 41.3, 40.3, 34.0, 33.2, 32.5, 32.4, 30.9, 28.7, 27.8, 26.0, 23.7, 23.5, 23.0, 22.2, 19.1, 18.7, 17.7. EIMS (70 eV) *m/z*: 532 (13) and 530 (14) $[M]^+$, 285 (5.6), 283 (6.2), 248 (100), 235 (10), 233 (11), 203 (84). HREIMS: Calcd for $C_{30}H_{43}O_3Br$: 530.2396. Found: 530.2383. Anal. (Table 2).

Methyl 2-Cyano-3-oxooleana-1,12-dien-28-oate (10). A solution of **27** (141 mg, 0.28 mmol) and DDQ (98%) (79 mg, 0.34 mmol) in benzene (10 mL) was heated under reflux for 4 h. After insoluble matter was removed by filtration, the filtrate was evaporated in vacuo to give a solid. The solid was subjected to flash column chromatography [benzene–acetone (20:1)] to give a crystalline solid (123 mg, 88%): mp 201–202 °C; $[\alpha]^{26}_D$ +67° (*c* 0.53, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 240 (3.65) nm. IR (KBr): 2945, 2874, 2232, 1724, 1686 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.75 (1H, s), 5.36 (1H, t, J = 3.5 Hz), 3.64 (3H, s), 2.91 (1H, dd, J = 3.9, 13.9 Hz), 1.22, 1.21, 1.15, 1.14, 0.94, 0.92, 0.83 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 198.3, 178.3, 170.2, 144.8, 121.1, 115.2, 114.0, 52.8, 51.8, 46.9, 45.8, 45.1, 42.3, 41.7, 41.3, 40.8, 40.5, 34.0, 33.3, 32.4, 32.3, 30.9, 27.9, 27.8, 26.0, 23.8, 23.4, 23.1, 21.8, 18.9, 18.1, 17.6. EIMS (70 eV) *m/z*: 491 $[M]^+$ (35), 459 (13), 432 (27), 262 (22), 247 (24), 203 (100). HREIMS: Calcd for $C_{32}H_{45}O_3N$: 491.3399. Found: 491.3391. Anal. (Table 2).

2-Cyano-3-oxooleana-1,12-dien-28-oic Acid (11). **11** was prepared from **10** according to the same method as for **B-15** except that the reaction time was 3 h. The reaction mixture was subjected to flash column chromatography [hexanes–EtOAc (3:1) followed by hexanes–EtOAc (2:1), then hexanes–EtOAc (1:1)] to give **11** as an amorphous solid (71%, 91% based on recovered **10**): $[\alpha]^{26}_D$ +61° (*c* 0.66, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 238 (3.87) nm. IR (KBr): 3387, 2947, 2870, 2233, 1729, 1691, 1609 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.75 (1H, s), 5.35 (1H, t, J = 3.3 Hz), 2.86 (1H, dd, J = 4.0, 13.6 Hz), 1.22, 1.21, 1.15, 1.12, 0.94, 0.92, 0.85 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 198.2, 184.3, 170.1, 144.4, 121.4, 115.1, 114.1, 52.7, 46.8, 45.7, 45.0, 42.2, 41.3, 40.8, 40.5, 33.9, 33.2, 32.5, 32.2, 30.9, 27.9, 27.7, 25.9, 23.7, 23.4, 22.9, 21.8, 18.9, 18.1, 17.7. EIMS (70 eV) *m/z*: 477 $[M]^+$ (18), 462 (5.6), 431 (16), 416 (10), 248 (76), 235 (25), 203 (100). HREIMS: Calcd for $C_{31}H_{43}O_3N$: 477.3243. Found: 477.3240. Anal. (Table 2).

Methyl 2-Cyano-3-oxoursa-1,12-dien-28-oate (12). **12** was prepared from **30** according to the same method as for **10** to give an amorphous solid (62%): $[\alpha]^{26}_D$ +53° (*c* 0.35, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 240 (3.74) nm. IR (KBr): 2973, 2926, 2870, 2229, 1723, 1686 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.77 (1H, s), 5.33 (1H, t, J = 3.7 Hz), 3.62 (3H, s), 2.29 (1H, d, J = 11.2 Hz), 1.23, 1.21, 1.14, 1.11 (each 3H, s), 0.96, 0.88 (each 3H, d, J = 6.3 Hz), 0.86 (3H, s). ^{13}C NMR ($CDCl_3$): δ 198.3, 178.1, 170.4, 139.3, 124.2, 115.2, 114.0, 53.2, 52.8, 51.7, 48.3, 45.1, 42.7, 41.2, 40.70, 40.65, 39.1, 39.0, 36.7, 32.6, 30.8, 28.1, 28.0, 24.3, 23.6, 23.4, 21.8, 21.3, 18.9, 18.2, 17.8, 17.2. EIMS (70

eV) *m/z*: 491 $[M]^+$ (38), 431 (35), 262 (46), 249 (82), 203 (65), 84 (100). HREIMS: Calcd for $C_{32}H_{45}O_3N$: 491.3399. Found: 491.3395. Anal. (Table 2).

2-Cyano-3-oxoursa-1,12-dien-28-oic Acid (13). **13** was prepared from **12** according to the same method as for **B-15** except that the reaction time was 4 h. The reaction mixture was subjected to prep-TLC [hexanes–EtOAc (1.5:1)] to give **13** as an amorphous solid (74%): $[\alpha]^{26}_D$ +48° (*c* 0.50, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 238 (3.86) nm. IR (KBr): 3417, 2973, 2926, 2870, 2233, 1731, 1689 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.77 (1H, s), 5.31 (1H, t, J = 3.2 Hz), 2.24 (1H, d, J = 11.0 Hz), 1.22, 1.20, 1.12, 1.11 (each 3H, s), 0.95, 0.88 (each 3H, d, J = 5.7 Hz), 0.87 (3H, s). ^{13}C NMR ($CDCl_3$): δ 198.2, 184.2, 170.2, 139.0, 124.4, 115.1, 114.1, 52.8, 52.7, 48.2, 45.0, 42.6, 41.2, 40.68, 40.65, 39.1, 39.0, 36.7, 32.5, 30.7, 28.1, 28.0, 24.1, 23.6, 23.3, 21.8, 21.3, 18.9, 18.2, 17.7, 17.2. EIMS (70 eV) *m/z*: 477 $[M]^+$ (22), 431 (23), 248 (100), 203 (48). HREIMS: Calcd for $C_{31}H_{43}O_3N$: 477.3243. Found: 477.3240. Anal. (Table 2).

Methyl 2-Formyl-3-oxooleana-1,12-dien-28-oate (14). To a stirred solution of phenylselenenyl chloride (98%) (161 mg, 0.82 mmol) in CH_2Cl_2 (7.2 mL) was added a solution of pyridine (75 mg, 0.95 mmol) in CH_2Cl_2 (1.0 mL) in an ice bath. After 15 min, a solution of **25** (204 mg, 0.41 mmol) in CH_2Cl_2 (2.0 mL) was added, and the mixture was stirred an additional 1 h. After the mixture was washed with 10% aqueous HCl solution (3 mL) twice, 30% H_2O_2 (0.4 mL) was added to the stirred mixture in the ice bath. After an additional 40 min, the mixture was worked up according to the standard method to give a solid (199 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (5:1)] to afford **25** (20 mg) and **14** as an amorphous solid (144 mg; 71%, 79% based on recovered **25**): $[\alpha]^{26}_D$ +12° (*c* 0.60, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 238 (3.85) nm. IR (KBr): 2946, 2867, 1724, 1703, 1673, 1610 cm^{-1}. ^1H NMR ($CDCl_3$): δ 10.00 (1H, s), 7.79 (1H, s), 5.37 (1H, t, J = 3.6 Hz), 3.63 (3H, s), 2.90 (1H, dd, J = 4.2, 13.9 Hz), 1.18, 1.17, 1.16, 1.14, 0.94, 0.91, 0.85 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 203.7, 190.7, 178.3, 165.2, 144.5, 131.2, 121.6, 52.8, 51.8, 47.0, 45.8, 45.1, 42.3, 41.7, 41.3, 40.5, 39.8, 34.0, 33.3, 32.44, 32.38, 30.9, 28.2, 27.8, 26.0, 23.8, 23.5, 23.2, 21.7, 19.2, 18.2, 17.6. EIMS (70 eV) *m/z*: 494 $[M]^+$ (95), 435 (87), 262 (40), 203 (100). HREIMS: Calcd for $C_{32}H_{46}O_4$: 494.3396. Found: 494.3398. Anal. (Table 2).

2-Formyl-3-oxooleana-1,12-dien-28-oic Acid (15). **15** was prepared from **32** according to the same method as for **14**. The reaction mixture was subjected to flash column chromatography [hexanes–EtOAc (3:1) followed by hexanes–EtOAc (2:1)] to give **15** as an amorphous solid (71%, 84% based on recovered **32**): $[\alpha]^{26}_D$ +26° (*c* 0.95, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 240 (3.82) nm. IR (KBr): 2948, 2866, 1725, 1701, 1674, 1608 cm^{-1}. ^1H NMR ($CDCl_3$): δ 10.00 (1H, s), 7.79 (1H, s), 5.36 (1H, t, J = 3.3 Hz), 2.86 (1H, dd, J = 3.8, 13.9 Hz), 1.18, 1.17, 1.15, 1.14, 0.94, 0.92, 0.87 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 203.7, 190.7, 184.3, 165.0, 144.2, 131.2, 121.8, 52.8, 46.8, 45.7, 45.1, 42.3, 41.4, 41.3, 40.5, 39.8, 34.0, 33.2, 32.5, 32.3, 30.9, 28.2, 27.8, 26.0, 23.7, 23.5, 23.0, 21.6, 19.2, 18.2, 17.8. EIMS (70 eV) *m/z*: 480 $[M]^+$ (5.5), 434 (3.1), 419 (3.4), 248 (56), 233 (27), 203 (100). HREIMS: Calcd for $C_{31}H_{44}O_4$: 480.3240. Found: 480.3237. Anal. (Table 2).

Methyl 2-Carboxy-3-oxooleana-1,12-dien-28-oate (16). **(1) From 14**: To a solution of **14** (357 mg, 0.72 mmol) in acetone (71 mL) was added Jones reagent (0.5 mL) dropwise in an ice bath. The mixture was stirred in the ice bath for 20 min. After excess Jones reagent was decomposed with MeOH, the acetone was evaporated in vacuo. After water was added to the resultant mixture, the aqueous mixture was extracted with EtOAc three times. The extract was washed with water (three times) and saturated aqueous NaCl solution (three times), dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated in vacuo to give a residue (294 mg). The residue was subjected to flash column chromatography [hexanes–EtOAc (1:1) followed by EtOAc] to afford **14** (89 mg) and **16** as a crystalline solid (109 mg; 30%, 39% based on recovered **14**): mp 230–231 °C; $[\alpha]^{26}_D$ +85° (*c* 0.61, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 234 (3.78) nm. IR (KBr): 3436, 2946, 2876, 1756, 1722, 1633 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.43 (1H, s), 5.36 (1H, t, J = 3.5 Hz), 3.64 (3H, s), 2.90 (1H, dd, J = 3.9, 13.7 Hz), 1.24, 1.21, 1.19, 1.13, 0.94, 0.91, 0.85 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 209.2, 178.4, 173.4, 165.2, 144.5, 123.3, 121.4, 52.4, 51.8, 47.0, 45.7, 45.5, 42.3, 41.7, 41.1, 40.6, 40.4, 34.0, 33.3, 32.4, 32.3, 30.9, 28.3, 27.8, 26.0, 23.8, 23.5, 23.1, 22.0, 19.0, 18.3, 17.7. EIMS (70 eV) *m/z*: 510 $[M]^+$ (16), 492 (15), 451 (14), 433 (14), 262 (27), 203 (100). HREIMS: Calcd for $C_{32}H_{46}O_5$: 510.3345. Found: 510.3347. Anal. (Table 2).

(2) From 17: A solution of **17** (500 mg, 0.95 mmol) in MeOH (29 mL) and aqueous KOH solution (KOH, 2.9 g; water, 10 mL) was heated under reflux for 15 min. After removal of MeOH in vacuo, the mixture was acidified with 5% aqueous HCl solution. It was extracted with EtOAc (three times). The extract was washed with water and saturated aqueous NaCl solution (each three times), dried over $MgSO_4$, and filtered. The filtrate gave **16** as a crystalline solid (470 mg, 97%). It was used for the next reaction without further purification.

Methyl 2-Methoxycarbonyl-3-oxooleana-1,12-dien-28-oate (17). **17** was prepared from **31** by the similar method as for **14**. The reaction mixture was subjected to flash column chromatography [hexanes–EtOAc (4:1)] to give **17** as an amorphous solid (83%, 90% based on recovered **31**): $[\alpha]^{26}_D$ +63° (*c* 0.78, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 230 (3.97) nm. IR (KBr): 2947, 2866, 1727, 1684, 1624 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.73 (1H, s), 5.37 (1H, t, J = 3.5 Hz), 3.79, 3.64 (each 3H, s), 2.90 (1H, dd, J = 3.9, 13.7 Hz), 1.16 (6H, s), 1.15, 1.12, 0.94, 0.91, 0.84 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 201.2, 178.4, 166.0, 164.3, 144.5, 129.2, 121.7, 52.7, 52.4, 51.8, 47.0, 45.9, 45.8, 42.3, 41.8, 41.5, 40.3, 39.5, 34.1, 33.3, 32.4, 32.3, 30.9, 28.7, 27.8, 25.9, 23.8, 23.6, 23.2, 21.5, 19.4, 18.0, 17.5. EIMS (70 eV) *m/z*: 524 $[M]^+$ (24), 492 (23), 465 (13), 262 (35), 203 (100). HREIMS: Calcd for $C_{33}H_{48}O_5$: 524.3502. Found: 524.3494. Anal. (Table 2).

2-Methoxycarbonyl-3-oxooleana-1,12-dien-28-oic Acid (18). A solution of **3** (52 mg, 0.10 mmol) in MeOH (5.2 mL) containing concentrated H_2SO_4 (0.15 mL) was heated under reflux for 30 min. After saturated aqueous NaCl solution was added to the mixture, it was extracted with EtOAc three times. The extract was worked up according to the standard method to give a residue (53 mg). The residue was subjected to flash column chromatography [hexanes–EtOAc (2:1)] to give **18** as an amorphous solid (42 mg, 78%): $[\alpha]^{26}_D$ +61° (*c* 0.56, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 230 (3.83) nm. IR (KBr): 3323, 2947, 2866, 1733, 1695, 1622 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.73 (1H, s), 5.37 (1H, t, J = 3.4 Hz), 3.79 (3H, s), 2.86 (1H, dd, J = 4.1, 13.7 Hz), 1.16, 1.15, 1.14, 1.12, 0.94, 0.92, 0.86 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 201.1, 184.2, 166.0, 164.2, 144.2, 129.2, 122.0, 52.7, 52.4, 46.9, 45.9, 45.8, 42.2, 41.5, 41.4, 40.3, 39.5, 34.0, 33.3, 32.5, 32.3, 30.9, 28.7, 27.8, 26.0, 23.7, 23.6, 23.0, 21.4, 19.4, 18.0, 17.7. EIMS (70 eV) *m/z*: 510 $[M]^+$ (2.6), 495 (2.0), 478 (2.5), 432 (3.0), 263 (29), 248 (58), 231 (37), 203 (100). HREIMS: Calcd for $C_{32}H_{46}O_5$: 510.3345. Found: 510.3344. Anal. (Table 2).

Methyl 2-Aminocarbonyl-3-oxooleana-1,12-dien-28-oate (19). A solution of **17** (100 mg, 0.19 mmol) in saturated ammonia MeOH (10 mL) was kept at room temperature overnight. The mixture was evaporated in vacuo to give a solid (108 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (1.5:1)] to give **19** as an amorphous solid (94 mg, 96%): $[\alpha]^{26}_D$ +77° (*c* 0.60, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 236 (3.91) nm. IR (KBr): 3413, 2943, 2866, 1727, 1689 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.45 (1H, brs), 8.27 (1H, s), 5.72 (1H, brs), 5.37 (1H, t, J = 3.4 Hz), 3.64 (3H, s), 2.90 (1H, dd, J = 4.2, 13.9 Hz), 1.17, 1.16, 1.15, 1.14, 0.94, 0.92, 0.84 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 205.8, 178.4, 169.0, 165.8, 144.3, 121.8, 52.2, 51.8, 47.0, 46.0, 45.7, 42.3, 41.8, 41.2, 40.4, 39.6, 34.1, 33.3, 32.5, 32.3, 30.9, 29.1, 27.8, 26.0, 23.8, 23.6, 23.2, 21.9, 19.4, 18.6, 17.6. EIMS (70 eV) *m/z*: 509 $[M]^+$ (34), 492 (23), 450 (100), 262 (19), 203 (56). HREIMS: Calcd for $C_{32}H_{47}O_4N$: 509.3505. Found: 509.3500. Anal. (Table 2).

Methyl 1α,2α-Epoxy-3-oxoolean-12-en-28-oate (22).[9] To a solution of **B-13** (223 mg, 0.48 mmol) in 2 N aqueous NaOH solution (1.7 mL) and THF (11 mL) was added a solution of

30% H_2O_2 (1.4 mL) in MeOH (2.8 mL) in an ice bath. The mixture was stirred at room temperature for 4 h. To the mixture were added saturated aqueous $NaHSO_3$ and 5% aqueous NaOH solutions, successively. After removal of THF and MeOH, the resultant mixture was acidified with 6 M aqueous HCl solution. The acidic layer was extracted with CH_2Cl_2 three times. The extract was worked up according to the standard method to give **22** as a crystalline solid (228 mg, 99%). This material was used for the next reaction without further purification. An analytically pure sample was obtained by recrystallization from MeOH as colorless needles: mp 212–213 °C; $[\alpha]^{26}_D$ +157° (*c* 0.80, $CHCl_3$). IR (KBr): 2943, 2866, 1727, 1699 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.36 (1H, t, J = 3.3 Hz), 3.64 (3H, s), 3.50 (1H, d, J = 4.5 Hz), 3.37 (1H, d, J = 4.5 Hz), 2.90 (1H, dd, J = 4.2, 13.9 Hz), 1.21, 1.11, 1.01, 0.97, 0.94, 0.92, 0.80 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 213.0, 178.4, 144.5, 121.8, 64.1, 57.1, 51.8, 47.0, 46.3, 45.9, 45.0, 42.1, 41.7, 40.8, 39.7, 38.8, 34.1, 33.3, 32.5, 32.3, 30.9, 28.2, 28.0, 26.0, 24.0, 23.8, 23.3, 21.1, 19.1, 17.4, 15.1. EIMS (70 eV) *m/z*: 482 $[M]^+$ (7.7), 422 (13), 262 (31), 249 (11), 203 (100). HREIMS: Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3391.

Methyl 2-Methoxy-3-oxooleana-1,12-dien-28-oate (23). A mixture of **22** (300 mg, 0.62 mmol) and Na (360 mg) in MeOH (36 mL) was heated under reflux for 48 h. After removal of MeOH in vacuo, the resultant mixture was diluted with water and then acidified with 6 M aqueous HCl solution. The aqueous mixture was extracted with a mixture of CH_2Cl_2 and Et_2O (1:2) three times. The extract was worked up according to the standard method to give a solid (320 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (4:1)] to afford **22** (31 mg) and **23** as an amorphous solid (270 mg; 87%, 98% based on recovered **22**): UV (EtOH) λ_{max} (log ϵ): 266 (3.77) nm. IR (KBr): 2946, 2866, 1727, 1682, 1621 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.96 (1H, s), 5.36 (1H, t, J = 3.5 Hz), 3.64, 3.55 (each 3H, s), 2.90 (1H, dd, J = 4.1, 13.7 Hz), 1.17 (6H, s), 1.16, 1.13, 0.93, 0.90, 0.81 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 200.1, 178.4, 149.0, 144.6, 126.3, 121.9, 54.9, 53.2, 51.8, 47.0, 45.9, 45.4, 43.3, 42.3, 41.7, 40.2, 38.4, 34.0, 33.3, 32.6, 32.5, 30.9, 28.5, 27.8, 26.0, 23.81, 23.76, 23.2, 22.0, 20.4, 19.2, 17.4. EIMS (70 eV) *m/z*: 496 $[M]^+$ (80), 436 (21), 328 (19), 262 (36), 203 (100). HREIMS: Calcd for $C_{32}H_{48}O_4$: 496.3553. Found: 496.3544.

Methyl 2-Hydroxy-3-oxooleana-1,12-dien-28-oate (24). A suspension of **23** (100 mg, 0.20 mmol) in 3 M aqueous HCl solution (3 mL) and AcOH (3 mL) was heated under reflux for 5 h. The mixture was neutralized with saturated aqueous Na_2CO_3 solution. The mixture was extracted with CH_2Cl_2 three times. The extract was worked up according to the standard method to give a solid (90 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (5:1)] to afford **24** as an amorphous solid (78 mg, 81%): UV (EtOH) λ_{max} (log ϵ): 272 (3.63) nm. IR (KBr): 3426, 2939, 2870, 1725, 1667, 1648 cm^{-1}. ^1H NMR ($CDCl_3$): δ 6.35 (1H, s), 5.93 (1H, brs), 5.34 (1H, t, J = 3.5 Hz), 3.63 (3H, s), 2.89 (1H, dd, J = 4.0, 13.7 Hz), 1.22 (6H, s), 1.13, 1.12, 0.94, 0.91, 0.80 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 201.3, 178.4, 144.4, 143.9, 128.4, 122.0, 54.1, 51.8, 47.0, 45.9, 44.1, 43.3, 42.2, 41.6, 40.2, 38.7, 34.1, 33.3, 32.7, 32.5, 30.9, 27.8, 27.4, 26.1, 23.8, 23.6, 23.2, 22.0, 19.8, 18.9, 17.5. EIMS (70 eV) *m/z*: 482 $[M]^+$ (26), 446 (68), 422 (25), 262 (35), 203 (100). HREIMS: Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3387.

Methyl 2-Hydroxymethylene-3-oxoolean-12-en-28-oate (25).[19] To a stirred mixture of **B-3** (1084 mg, 2.31 mmol) and ethyl formate (97%) (707 mg, 9.26 mmol) in benzene (12 mL) was added NaOMe (501 mg, 9.27 mmol). The mixture was stirred at room temperature for 1 h. After the mixture was washed with 5% aqueous HCl solution twice, it was worked up according to the standard method to give **25** as an amorphous solid (1095 mg, 95%). This material was used for the next reaction without further purification. An analytically pure sample was obtained by flash column chromatography [hexanes–EtOAc (7:1)] and subsequent recrystallization from MeOH as colorless needles: mp 199–201 °C. UV (EtOH) λ_{max} (log ϵ): 296 (3.94) nm. IR (KBr): 3426, 2943, 2862, 1725, 1637, 1588 cm^{-1}. ^1H NMR ($CDCl_3$): δ 14.92 (1H, d, J = 3.1 Hz), 8.58 (1H, d, J = 3.1 Hz), 5.35 (1H, t, J = 3.7 Hz), 3.64 (3H, s), 2.90 (1H, dd, J = 4.2, 13.6 Hz), 2.29 (1H, d, J = 14.4 Hz), 1.92 (1H, d, J = 14.4 Hz), 1.20, 1.16, 1.12, 0.94 (each 3H, s), 0.91 (6H, s), 0.80 (3H, s). ^{13}C NMR ($CDCl_3$): δ 190.9, 188.6, 178.4, 144.0, 122.3, 106.0, 52.3, 51.8, 47.0, 46.0, 45.9, 42.0, 41.6, 40.3, 39.4, 39.3, 36.5, 34.1, 33.3, 32.5, 32.1, 30.9, 28.6, 27.9, 25.9, 23.8, 23.6, 23.3, 21.1, 19.7, 16.8, 14.7. EIMS (70 eV) *m/z*: 496 $[M]^+$ (4.4), 437 (23), 262 (38), 233 (20), 203 (100). HREIMS: Calcd for $C_{32}H_{48}O_4$: 496.3553. Found: 496.3550.

Methyl Isoxazolo[4,5-*b*]olean-12-en-28-oate (26). A mixture of **25** (994 mg, 2.0 mmol), hydroxylamine hydrochloride (1391 mg, 20 mmol) in water (1.8 mL) and EtOH (48 mL) was heated under reflux for 1 h. After EtOH was removed in vacuo, EtOAc was added to the resultant mixture. The EtOAc layer was washed with water (three times) and saturated aqueous NaCl solution (three times), dried over $MgSO_4$, and filtered. The filtrate gave a solid (1086 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (6:1) followed by hexanes–EtOAc (5:1)] to give **26** as an amorphous solid (934 mg, 86%): UV (EtOH) λ_{max} (log ϵ): 228 (3.65) nm. IR (KBr): 2940, 2864, 1725 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.98 (1H, s), 5.34 (1H, t, J = 3.5 Hz), 3.63 (3H, s), 2.89 (1H, dd, J = 4.4, 13.7 Hz), 2.42 (1H, d, J = 15.1 Hz), 1.30, 1.21, 1.15, 0.93, 0.90, 0.88, 0.79 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 178.4, 173.2, 150.4, 144.0, 122.3, 109.0, 53.7, 51.7, 46.9, 46.3, 46.0, 42.0, 41.6, 39.5, 38.9, 35.5, 34.9, 34.0, 33.3, 32.5, 32.1, 30.9, 29.0, 27.9, 25.9, 23.8, 23.5, 23.2, 21.6, 19.0, 16.7, 15.4. EIMS (70 eV) *m/z*: 493 $[M]^+$ (11), 434 (18), 262 (28), 249 (16), 203 (100). HREIMS: Calcd for $C_{32}H_{47}O_3N$: 493.3556. Found: 493.3556.

Methyl 2-Cyano-3-oxoolean-12-en-28-oate (27). To a stirred solution of **26** (887 mg, 1.80 mmol) in Et_2O (50 mL) and MeOH (25 mL) was added NaOMe (3.2 g) in an ice bath. The mixture was stirred at room temperature for 1 h. The mixture was diluted with a mixture of CH_2Cl_2 and Et_2O (1:2) (50 mL). After the extract was washed with 5% aqueous HCl solution, it was worked up according to the standard method to afford **27** as an amorphous solid (879 mg, 99%). This material was used for the next reaction without further purification. An analytically pure sample was obtained by flash column chromatography [hexanes–EtOAc (5:1)] as an amorphous solid: UV (EtOH) λ_{max} (log ϵ): 238 (3.88) nm. IR (KBr): 2946, 2870, 2202, 1724, 1633 cm^{-1}. ^1H NMR of major tautomer **27a** ($CDCl_3$): δ 6.15 (1H, brs), 5.31 (1H, t, J = 3.6 Hz), 3.63 (3H, s), 2.88 (1H, dd, J = 4.0, 13.6 Hz), 2.09 (1H, d, J = 15.0 Hz), 1.16, 1.13, 1.07, 0.95, 0.93, 0.90, 0.76 (each 3H, s). EIMS (70 eV) *m/z*: 493 $[M]^+$ (6.3), 434 (17), 262 (19), 249 (20), 203 (100). HREIMS: Calcd for $C_{32}H_{47}O_3N$: 493.3556. Found: 493.3548.

Methyl 2-Hydroxymethylene-3-oxours-12-en-28-oate (28).[23] **28** was prepared from **B-4** according to the same method as for **25** to give an amorphous solid (quantitative). This material was used for the next reaction without further purification. An analytically pure sample was obtained by flash column chromatography [hexanes–EtOAc (7:1)] and subsequent recrystallization from MeOH as colorless needles: mp 170–171 °C. UV (EtOH) λ_{max} (log ϵ): 294 (3.86) nm. IR (KBr): 3426, 2947, 2921, 2866, 1727, 1637, 1590 cm^{-1}. ^1H NMR ($CDCl_3$): δ 14.91 (1H, brs), 8.57 (1H, s), 5.31 (1H, t, J = 3.7 Hz), 3.62 (3H, s), 2.31 (1H, d, J = 14.4 Hz), 2.27 (1H, d, J = 12.5 Hz), 1.95 (1H, d, J = 14.4 Hz), 1.19, 1.12, 1.10 (each 3H, s), 0.96 (3H, d, J = 6.0 Hz), 0.92 (3H, s), 0.87 (3H, d, J = 6.6 Hz), 0.81 (3H, s). ^{13}C NMR ($CDCl_3$): δ 191.0, 188.5, 178.2, 138.4, 125.6, 106.0, 53.2, 52.3, 51.7, 48.4, 45.7, 42.4, 40.3, 39.7, 39.5, 39.3, 39.1, 36.8, 36.4, 32.4, 30.9, 28.7, 28.2, 24.4, 23.7, 23.6, 21.4, 21.1, 19.7, 17.2, 17.0, 14.8. EIMS (70 eV) *m/z*: 496 $[M]^+$ (11), 437 (15), 262 (80), 233 (41), 203 (100). HREIMS: Calcd for $C_{32}H_{48}O_4$: 496.3553. Found: 496.3547.

Methyl Isoxazolo[4,5-*b*]urs-12-en-28-oate (29). 29 was prepared from **28** according to the same method as for **26** to give an amorphous solid (84%): UV (EtOH) λ_{max} (log ϵ): 228 (3.70) nm. IR (KBr): 2969, 2922, 2870, 1725 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.98 (1H, s), 5.31 (1H, t, J = 3.4 Hz), 3.62 (3H, s), 2.46 (1H, d, J = 15.0 Hz), 2.27 (1H, d, J = 11.1 Hz), 1.31, 1.22,

1.10 (each 3H, s), 0.96 (3H, d, $J = 6.3$ Hz), 0.90 (3H, s), 0.88 (3H, d, $J = 6.3$ Hz), 0.81 (3H, s). ^{13}C NMR ($CDCl_3$): δ 178.2, 173.2, 150.4, 138.4, 125.5, 109.1, 53.7, 53.2, 51.7, 48.3, 46.3, 42.3, 39.7, 39.3, 39.1, 38.8, 36.8, 35.8, 34.9, 32.4, 30.9, 29.1, 28.3, 24.4, 23.7, 23.5, 21.6, 21.4, 19.0, 17.2, 16.9, 15.6. EIMS (70 eV) *m/z*: 493 $[M]^+$ (9.1), 434 (20), 262 (65), 249 (33), 203 (100). HREIMS: Calcd for $C_{32}H_{47}O_3N$: 493.3556. Found: 493.3547.

Methyl 2-Cyano-3-oxours-12-en-28-oate (30). **30** was prepared from **29** according to the same method as for **27** to give an amorphous solid (quantitative). This material was used for the next reaction without further purification. An analytically pure sample was obtained by flash column chromatography [hexanes−EtOAc (5:1)] as an amorphous solid: UV (EtOH) λ_{max} (log ϵ): 238 (3.93) nm. IR (KBr): 2947, 2870, 2203, 1724, 1631 cm^{-1}. ^{1}H NMR of major tautomer **30a** ($CDCl_3$): δ 5.92 (1H, brs), 5.28 (1H, t, $J = 3.5$ Hz), 3.61 (3H, s), 2.26 (1H, d, $J = 11.0$ Hz), 2.13 (1H, d, $J = 15.0$ Hz), 1.16, 1.13, 1.08, 1.07, 0.96 (each 3H, s), 0.95, 0.77 (each 3H, d, $J = 6.3$ Hz). EIMS (70 eV) *m/z*: 493 $[M]^+$ (6.8), 434 (19), 262 (62), 249 (44), 203 (100). HREIMS: Calcd for $C_{32}H_{47}O_3N$: 493.3556. Found: 493.3558.

Methyl 3-Hydroxy-2-methoxycarbonyloleana-2,12-dien-28-oate (31). A mixture of **B-3** (2.0 g, 4.27 mmol) and 1.8 M DMF solution of methoxymagnesium methyl carbonate (Stiles' reagent) (20 mL, 36 mmol) was heated under reflux for 2 h while a slow stream of N_2 was bubbled through the mixture with a pipet. To the mixture were added 5% aqueous HCl solution and EtOAc. The aqueous layer was extracted with EtOAc (three times). The combined organic layers were washed with water (three times) and saturated aqueous NaCl solution (three times), dried over $MgSO_4$, and filtered. The filtrate was evaporated in vacuo to give a solid (2.26 g). To a solution of the solid in THF (30 mL) was added excessive amount of ethereal diazomethane. The mixture was kept at room temperature for 10 min. The mixture was evaporated in vacuo to give a solid (2.38 g). The solid was subjected to flash column chromatography [hexanes−EtOAc (7:1)] to give **B-3** (330 mg) and **31** as crystals (1.66 g; 74%, 89% based on recovered **B-3**): mp 160−162 °C. UV (EtOH) λ_{max} (log ϵ): 262 (4.01) nm. IR (KBr): 2948, 2858, 1737, 1660, 1615 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 12.51 (1H, s), 5.33 (1H, t, $J = 3.7$ Hz), 3.74, 3.63 (each 3H, s), 2.89 (1H, dd, $J = 4.2$, 13.9 Hz), 2.35 (1H, d, $J = 15.7$ Hz), 1.18, 1.14, 1.10, 0.94 (each 3H, s), 0.91 (6H, s), 0.78 (3H, s). ^{13}C NMR ($CDCl_3$): δ 178.5, 177.9, 174.2, 143.8, 122.6, 94.3, 52.5, 51.8, 51.7, 47.0, 46.13, 46.09, 42.0, 41.7, 39.4, 38.6, 38.4, 35.7, 34.1, 33.3, 32.6, 32.1, 31.0, 28.8, 27.9, 26.0, 23.8, 23.6, 23.3, 20.4, 19.8, 16.8, 15.1. EIMS (70 eV) *m/z*: 526 $[M]^+$ (0.6), 494 (5.6), 479 (2.5), 466 (1.6), 435 (13), 262 (28), 203 (100). HREIMS: Calcd for $C_{33}H_{50}O_5$: 526.3658. Found: 526.3658.

2-Hydroxymethylene-3-oxoolean-12-en-28-oic Acid (32). To a stirred mixture of oleanonic acid (**B-1**)[10] (540 mg, 1.19 mmol) and ethyl formate (97%) (357 mg, 4.66 mmol) in THF (12 mL) was added NaOMe (258 mg, 4.78 mmol). The mixture was stirred at room temperature overnight. The mixture was acidified with 10% aqueous HCl solution. The mixture was extracted with EtOAc three times. The extract was worked up according to the standard method to give a solid (600 mg). The solid was subjected to flash column chromatography [hexanes−EtOAc (5:1) followed by hexanes−EtOAc (4:1)] to afford **B-1** (168 mg) and **32** as a crystalline solid (260 mg; 45%, 66% based on recovered **B-1**): mp 200−203 °C dec. UV (EtOH) λ_{max} (log ϵ): 292 (3.93) nm. IR (KBr): 2946, 2654, 1732, 1694, 1644, 1587 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 14.91 (1H, brs), 8.59 (1H, s), 5.34 (1H, t, $J = 3.5$ Hz), 2.86 (1H, dd, $J = 4.5$, 13.9 Hz), 2.29 (1H, d, $J = 14.6$ Hz), 1.93 (1H, d, $J = 14.6$ Hz), 1.19, 1.16, 1.10, 0.94, 0.92, 0.91, 0.82 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 190.7, 188.8, 184.7, 143.8, 122.6, 105.9, 52.2, 46.8, 46.0, 45.9, 41.9, 41.2, 40.2, 39.34, 39.30, 36.5, 34.0, 33.3, 32.6, 32.0, 30.9, 28.6, 27.8, 25.9, 23.7, 23.5, 23.1, 21.0, 19.6, 17.0, 14.6. EIMS (70 eV) *m/z*: 482 $[M]^+$ (1.8), 438 (2.7), 436 (3.6), 248 (77), 203 (100). HREIMS: Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3392.

Evaluation Methods. 1. Reagents. Recombinant mouse IFN-γ (LPS content, <10 pg/mL) was purchased from Genzyme (Cambridge, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Inhibitory test compounds were dissolved in DMSO before addition to cell cultures; final concentrations of DMSO were 0.1% or less. Controls with DMSO alone were run in all cases.

2. Cell Culture. To obtain primary macrophages, female CD-1 mice, 5−10 weeks of age (Charles River Breeding Laboratories, Wilmington, MA), were injected intraperitoneally with 2 mL of 4% thioglycollate broth (Difco Laboratories, Detroit, MI). Four days after injection, peritoneal macrophages were harvested and processed according to Nathan's procedure.[7b] Cells were seeded in 96-well plates at 2×10^5 cells/well and incubated for 48 h with 20 ng/mL IFN-γ in the presence or absence of inhibitory test compounds.

3. Measurement of NO Production in Mouse Macrophages. Nitrite accumulation was used as an indicator of NO production in the medium and was assayed by the Griess reaction.[7a] Griess reagent (100 μL) was added to 100 μL of each supernatant from IFN-γ or inhibitory test compound-treated cells in triplicate. The protein determination was performed by Bradford protein assay. The plates were read at 550 nm against a standard curve of sodium nitrite.

Acknowledgment. We thank Drs. Carl Nathan and Qiao-wen Xie for expert advice on the preparation of macrophages and the nitric oxide assay. We also thank Dr. Steven Mullen (University of Illinois) for the mass spectra and Professor David A. Evans and Mr. Brett D. Allison (Harvard University) for the optical rotation measurements. This investigation was supported by funds from NIH Grant 1 R01-CA78814; the Norris Cotton Cancer Center; U.S. Department of Defense Grants DAMD17-96-1-6163, DAMD17-98-1-8604, and DAMD17-99-1-9168; the Oliver and Jennie Donaldson Charitable Trust; the National Foundation for Cancer Research; and a Zenith Award from the Alzheimer's Association. M.B.S. is an Oscar M. Cohn Professor, F.G.F. is an Oscar M. Cohn Scholar, and Y.W. is a Howard Hughes Medical Institute Predoctoral Fellow.

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JM000008J

Synthetic Oleanane and Ursane Triterpenoids with Modified Rings A and C: A Series of Highly Active Inhibitors of Nitric Oxide Production in Mouse Macrophages†

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Received May 26, 2000

We have designed and synthesized 16 new olean- and urs-1-en-3-one triterpenoids with various modified rings C as potential antiinflammatory and cancer chemopreventive agents and evaluated their inhibitory activities against production of nitric oxide induced by interferon-γ in mouse macrophages. This investigation revealed that 9(11)-en-12-one and 12-en-11-one functionalities in ring C increase the potency by about 2−10 times compared with the original 12-ene. Subsequently, we have designed and synthesized novel olean- and urs-1-en-3-one derivatives with nitrile and carboxyl groups at C-2 in ring A and with 9(11)-en-12-one and 12-en-11-one functionalities in ring C. Among them, we have found that methyl 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (**25**), 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) (**26**), and methyl 2-carboxy-3,12-dioxooleana-1,9(11)-dien-28-oate (**29**) have extremely high potency (IC_{50} = 0.1 nM level). Their potency is similar to that of dexamethasone although they do not act through the glucocorticoid receptor. Overall, the combination of modified rings A and C increases the potency by about 10 000 times compared with the lead compound, 3-oxooleana-1,12-dien-28-oic acid (**8**) (IC_{50} = 1 μM level). The selected oleanane triterpenoid, CDDO (**26**), was found to be a potent, multifunctional agent in various in vitro assays and to show antiinflammatory activity against thioglycollate−interferon-γ-induced mouse peritonitis.

Introduction

Oleanane and ursane triterpenoids are pentacyclic compounds with 30 carbon atoms, biosynthetically derived from the cyclization of squalene.[1] This is a vast class of natural products whose structural diversity includes a wide array of functional groups.[2] Many compounds of this group are reported to have various interesting biological, pharmacological, or medicinal activities including antiinflammatory and anticarcinogenic activities.[3] However, in many cases, the potency of these triterpenoids is relatively weak. Therefore, anticipating highly potent novel structures, we began bioassay-directed systematic drug design and synthesis of derivatives of commercially available oleanolic acid (**1**) and ursolic acid (**2**) (cf. Scheme 1).

To discover antiinflammatory and cancer chemopreventive drugs from these derivatives, we have adopted an assay system that measures inhibition of nitric oxide (NO) production induced by interferon-γ (IFN-γ) in mouse macrophages[4] as a preliminary screening assay system. In a previous paper,[5] we reported that olean-12-ene triterpenoids with a 1-en-3-one functionality having nitrile, methoxycarbonyl, and carboxyl groups at C-2 in ring A, **3**−**7**, show significant potency [IC_{50} = 0.01−0.1 μM level, about 10−100 times more potent than the lead compound **8** (IC_{50} = 1 μM level)] in this assay. As a continuation of this work, we have synthesized 16 new olean- and urs-1-en-3-one derivatives with various modified rings C, **9**−**24**, and evaluated their inhibitory activities in the above assay. This investigation revealed that 9(11)-en-12-one, 12-en-11-one, and 13-(18)-en-11-one functionalities in ring C increase the potency by about 2−10 times compared with the original 12-ene. Subsequently, we have designed and synthesized novel olean- and urs-1-en-3-one derivatives with nitrile, methoxycarbonyl, and carboxyl groups at C-2 in ring A and with 9(11)-en-12-one, 12-en-11-one, and 13-(18)-en-11-one functionalities in ring C, **25**−**35**. Among them, we have found that methyl 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (**25**), 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) (**26**), and methyl 2-carboxy-3,12-dioxooleana-1,9(11)-dien-28-oate (**29**) have extremely high potency (IC_{50} = 0.1 nM level). We report here the synthesis, inhibitory activity, and

† Part of this work has been reported in preliminary form: (a) Honda, T.; Finlay, H. J.; Gribble, G. W.; Suh, N.; Sporn, M. B. New enone derivatives of oleanolic acid and ursolic acid as inhibitors of nitric oxide production in mouse macrophages. *Bioorg. Med. Chem. Lett.* **1997**, *7*, 1623−1628. (b) Honda, T.; Rounds, B. V.; Gribble, G. W.; Suh, N.; Wang, Y.; Sporn, M. B. Design and synthesis of 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid, a novel and highly active inhibitor of nitric oxide production in mouse macrophages. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2711−2714. (c) Honda, T.; Rounds, B. V.; Bore, L.; Favaloro, F. G., Jr.; Gribble, G. W.; Suh, N.; Wang, Y.; Sporn, M. B. Novel synthetic oleanane triterpenoids: a series of highly active inhibitors of nitric oxide production in mouse macrophages. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 3429−3434.

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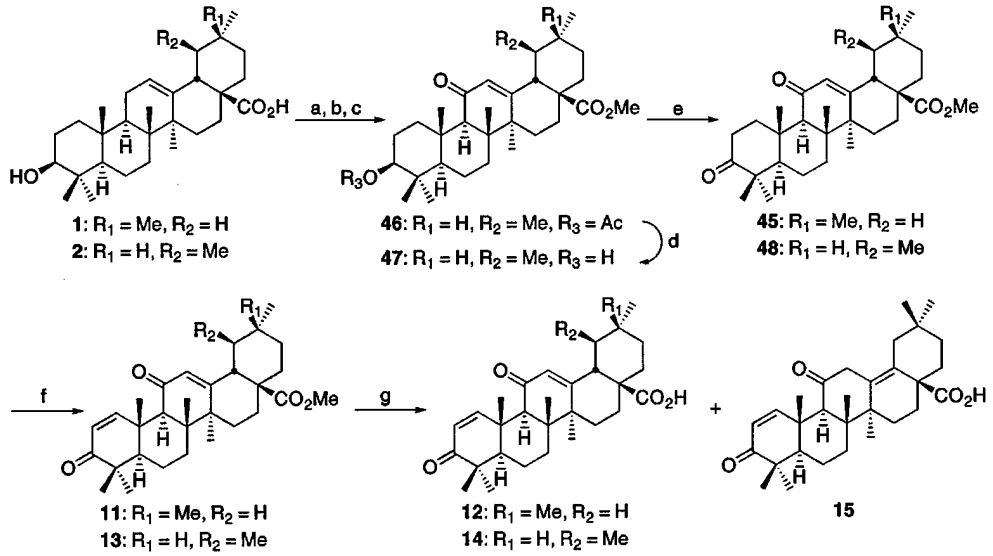
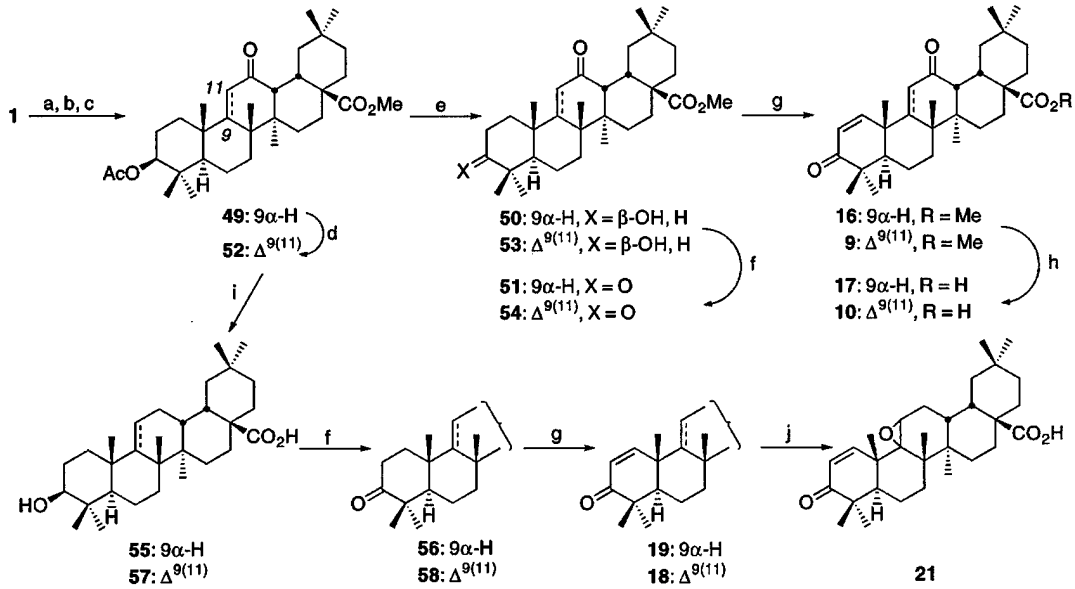
10.1021/jm0002230 CCC: $19.00 © 2000 American Chemical Society
Published on Web 09/13/2000

Scheme 1[a]



[a] Reagents: (a) CH_2N_2, Et_2O, THF; (b) Ac_2O, pyr; (c) CrO_3, pyr, CH_2Cl_2; (d) KOH, aq MeOH; (e) Jones; (f) PhSeCl, EtOAc, 30% H_2O_2, THF; (g) LiI, DMF.

Scheme 2[a]



[a] Reagents: (a) CH_2N_2, Et_2O, THF; (b) Ac_2O, pyr; (c) 30% H_2O_2, AcOH; (d) Br_2, HBr, AcOH; (e) KOH, aq MeOH; (f) Jones; (g) PhSeCl, EtOAc, 30% H_2O_2, THF; (h) LiI, DMF; (i) NH_2NH_2, KOH, diethylene glycol; (j) *m*CPBA, CH_2Cl_2.

structure–activity relationships (SARs) of these novel triterpenoids in detail.

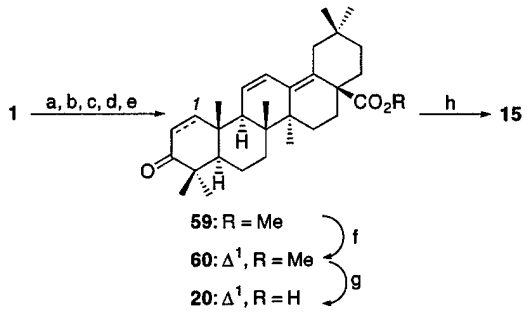
Chemistry

Modification of Ring C and Carboxyl Group at C-17. Enones **9**–**21** were designed and synthesized to discover what structures of ring C enhance potency in comparison with the original 12-ene, i.e., the lead compound **8**[5] (Schemes 1–3).[6] In addition, enones **22**–**24** were designed and synthesized to learn which functionality at C-17 is most appropriate (Scheme 4).

Enone **11** was prepared by introduction of a double bond at C-1 of known C-3 ketone **45**,[7] which was prepared in five steps from oleanolic acid (**1**), with phenylselenenyl chloride in ethyl acetate and sequential addition of 30% hydrogen peroxide ($PhSeCl–H_2O_2$) (yield, 97%).[8] Halogenolysis of **11** with lithium iodide (LiI) in *N,N*-dimethylformamide (DMF)[9] gave α,β- and β,γ-unsaturated ketones **12** and **15** in 43% and 22% yield, respectively. C-3 alcohol **47** was obtained quantitatively by alkaline hydrolysis (reflux) of known acetate **46**,[10] which was prepared in three steps from ursolic acid (**2**). Jones oxidation of **47** gave C-3 ketone **48** in 89% yield. Enone **13** was prepared in 93% yield from **48** by the same method as for **11**. Halogenolysis of **13** gave acid **14** in 58% yield.[11]

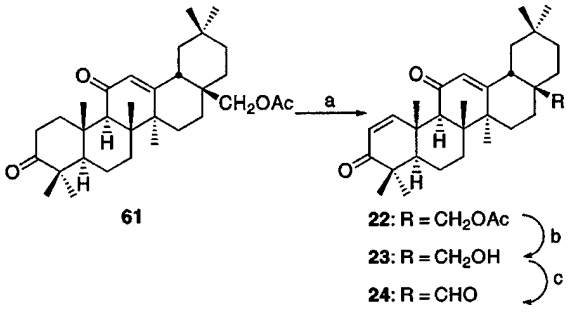
Similarly, enone **16** was synthesized in 74% yield via **50** and **51** from C-12 ketone **49**, which was prepared in three steps from **1** according to a known method,[12,13] and enone **9** was also synthesized in 60% yield via **53** and **54** from known C-12 ketone **52** which was prepared from **49** with bromine and hydrobromic acid in acetic acid.[14] Halogenolysis of enones **16** and **9** gave acids **17** and **10** in 62% and 68% yield, respectively. Enone **19**

Scheme 3[a]



[a] Reagents: (a) CH_2N_2, Et_2O, THF; (b) Ac_2O, pyr; (c) SeO_2, AcOH; (d) KOH, aq MeOH; (e) CrO_3, pyr, CH_2Cl_2; (f) PhSeCl, EtOAc, 30% H_2O_2, THF; (g) LiI, DMF; (h) Jones.

Scheme 4[a]



[a] Reagents: (a) PhSeCl, EtOAc, 30% H_2O_2, THF; (b) KOH, aq MeOH; (c) CrO_3, pyr, CH_2Cl_2.

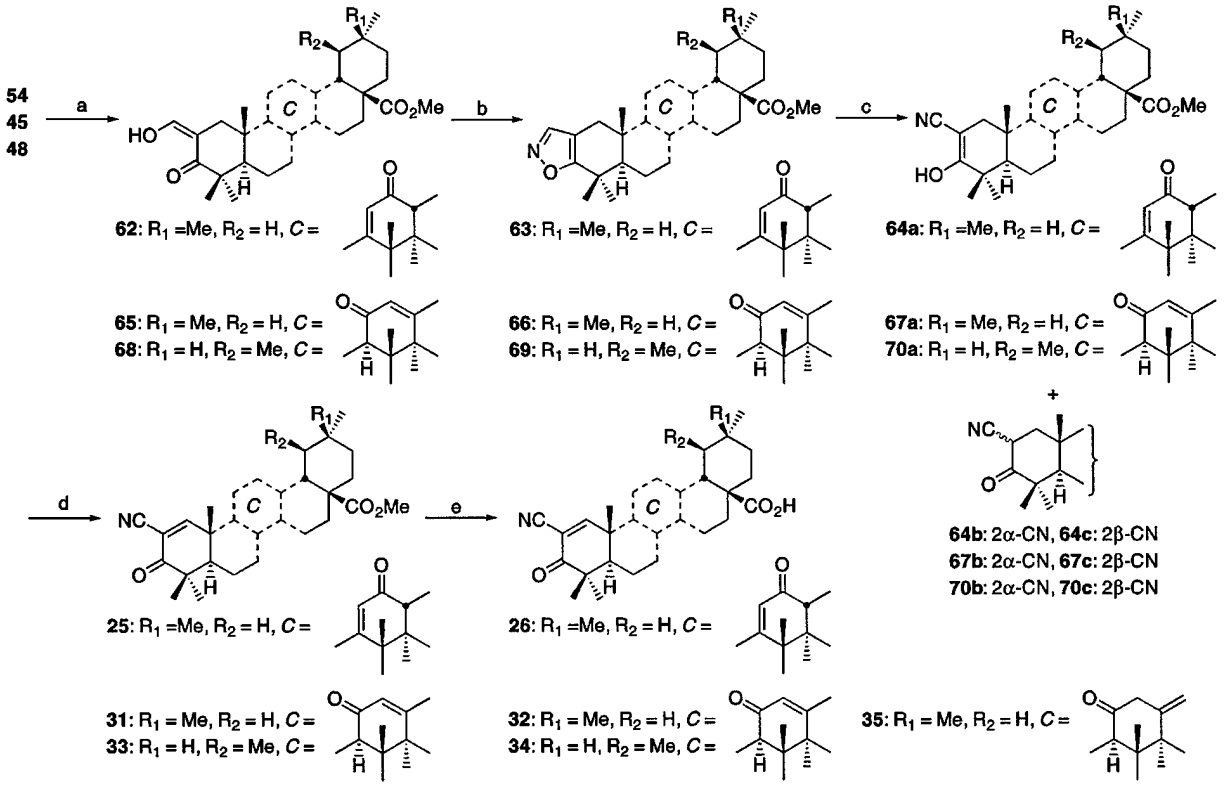
was obtained in 68% yield from known C-3 ketone **56**[15] which was synthesized via **55** from **49**. Enone **18** was obtained in 76% yield via **58** from acid **57**, which was prepared in 53% yield from **52** by Wolff–Kishner reduction. Epoxide **21**[16] was prepared in 46% yield by oxidation of **18** with *m*-chloroperbenzoic acid (*m*CPBA) in methylene chloride (CH_2Cl_2). Enone **20** was synthesized in 37% yield via **60** from known diene **59**[17] which was prepared in five steps from **1**. Interestingly, Jones oxidation of **20** afforded the same deconjugated enone **15** (yield, 28%) as halogenolysis of **11**. Enone **22** was prepared in 83% yield from krukovine A acetate (**61**), which was previously synthesized in our laboratory.[18] Alkaline hydrolysis (at room temperature)[19] of **22** gave enone **23** in 78% yield. Ratcliffe oxidation[20] of **23** with chromium trioxide and pyridine in CH_2Cl_2 afforded aldehyde **24** in 89% yield.

Among these new synthetic enones, **9**–**12** and **15** showed more inhibitory activity than the lead compound **8** on production of NO-induced IFN-γ in mouse macrophages (see Table 1). Overall, 9(11)-en-12-one, 12-en-11-one, and 13(18)-en-11-one functionalities in ring C increase the potency by about 2–10 times compared with the original 12-ene.

Combination of Modified Ring A with Ring C. On the basis of our previous results,[5] in which olean-12-ene triterpenoids with a 1-en-3-one functionality having nitrile, methoxycarbonyl, and carboxyl groups at C-2 in ring A, **3**–**7**, are about 10–100 times more potent than **8** (see Table 1), and the above results, we have designed and synthesized novel oleanane and ursane triterpenoids with modified rings A and C, **25**–**35**. In addition, to further discern SARs, amide **36** and enal **37** were designed and synthesized because amide **41** and enal **42** showed low potency and toxicity, respectively, in our previous evaluation (see Table 1).[5] The syntheses of these newly designed triterpenoids are illustrated in Schemes 5–7.

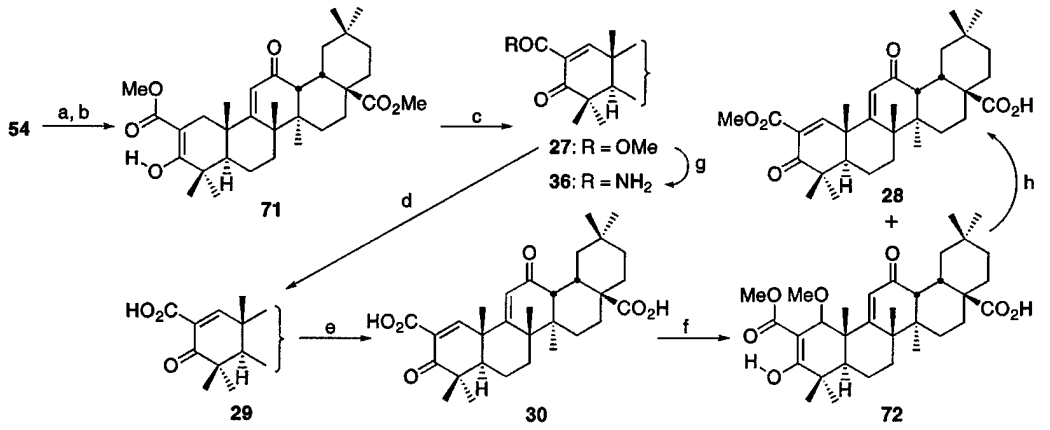
Hydroxymethylene **62**[21] was prepared in 99% yield by formylation of **54** with ethyl formate in the presence

Scheme 5[a]



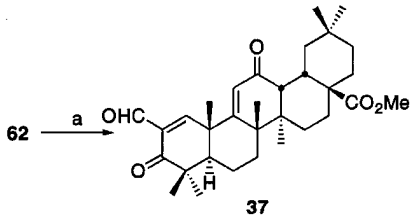
[a] Reagents: (a) HCO_2Et, NaOMe, PhH; (b) NH_2OH·HCl, aq EtOH; (c) NaOMe, Et_2O, MeOH; (d) DDQ, PhH; (e) LiI, DMF.

Scheme 6[a]



[a] Reagents: (a) Stiles' reagent, DMF; (b) CH_2N_2, Et_2O, THF; (c) PhSeCl, pyr, CH_2Cl_2, 30% H_2O_2, CH_2Cl_2; (d) KOH, aq MeOH; (e) LiI, DMF; (f) H_2SO_4, MeOH; (g) NH_3, MeOH; (h) SiO_2.

Scheme 7[a]



[a] Reagents: (a) PhSeCl, pyr, CH_2Cl_2, 30% H_2O_2, CH_2Cl_2.

of sodium methoxide in benzene.[22] Isoxazole **63** was obtained in 66% yield from **62** by the addition of hydroxylamine.[23] Cleavage of the isoxazole moiety of **63** with sodium methoxide gave nitrile **64** quantitatively.[23,24] CDDO methyl ester (**25**) was prepared in 92% yield by 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) oxidation of **64** in benzene, although PhSeCl–H_2O_2 gave **25** in only 40% yield. Halogenolysis of **25** gave CDDO (**26**) in 68% yield. Similarly, olean-12-en-11-one derivative **31** was synthesized in 53% yield via **65**,[21] **66**, and **67**[24] from **45**. Halogenolysis of **31** gave α,β- and β,γ-unsaturated ketones **32** and **35** in 37% and 16% yield, respectively. Urs-12-en-11-one derivative **33** was also synthesized in 61% yield via **68**,[21] **69**, and **70**[24] from **48**. Halogenolysis of **33** gave acid **34** in 60% yield.[11]

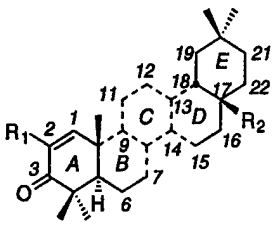
Ester **71** was prepared in 78% yield from C-3 ketone **54** by Stiles' reagent (methoxymagnesium methyl carbonate) in DMF,[25] followed by methylation with diazomethane. ^{1}H NMR showed that **71** in $CDCl_3$ is the single tautomer depicted in Scheme 6. Enone **27** was prepared from **71** by PhSeCl–pyridine in CH_2Cl_2 and sequential addition of 30% H_2O_2[26] (yield, 71%; 88% based on recovered **71**). Hydrolysis (reflux) of **27** with potassium hydroxide in aqueous methanol (MeOH) gave C-2 carboxylic acid **29** and decarboxylated enone **9** in 78% and 8% yield, respectively. Because of the steric hindrance of the methoxycarbonyl group at C-17 of **27**, the above conditions gave monoesters **29** and **9** selectively. Halogenolysis of **29** gave dicarboxylic acid **30** and decarboxylated enone **10** in 47% and 24% yield, respectively. Interestingly, methylation of **30** with MeOH under acidic conditions gave a mixture of desired monoester **28** and Michael adduct **72**.[27] The ratio of **28** to **72** was determined to be 4:5 by ^{1}H NMR. Because the adduct **72** was readily transformed into **28** under purification conditions (see Experimental Section), **28** was finally obtained in 82% yield from **30**. Amide **36** was prepared selectively from **27** with saturated ammonia–MeOH (yield, 49%; 88% based on recovered **27**). Enal **37** was synthesized from **62** according to the same method as for **27** (yield, 62%; 74% based on recovered **62**).

Biological Results and Discussion

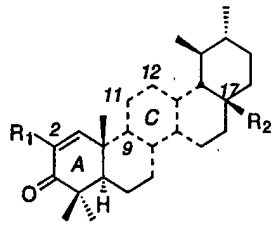
The inhibitory activities [IC_{50} (μM) value] of synthetic triterpenoids **3**–**44**, oleanolic acid (**1**), ursolic acid (**2**), hydrocortisone, and dexamethasone (both glucocorticoids are used as positive controls) on NO production induced by IFN-γ in mouse macrophages are shown in Table 1. These derivatives are arranged categorically in order of the amplification of potency due to the structure of ring C. Among novel synthetic oleanane and ursane triterpenoids, **25**, CDDO (**26**), and **29** showed extremely high potency (IC_{50} = 0.1 nM level). Their potency is equivalent to that of dexamethasone although their inhibitory activity is not blocked by the glucocorticoid antagonist, RU-486,[28] which reverses the action of dexamethasone (data not shown).

This series of synthetic triterpenoids showed the following interesting SARs: (1) A 9(11)-en-12-one functionality is the strongest enhancer of potency among structures of ring C. Oleanane triterpenoids **10** and **9** (IC_{50} = 0.1 μM level) are about 10–100 times more potent than the lead compounds **8** (IC_{50} = 1 μM level) and **43** (IC_{50} = 10 μM level), respectively. (2) 12-En-11-one, 13(18)-en-11-one, and 12-one functionalities also enhance potency. Oleanane triterpenoids **11**, **12**, **15**, and **17** are more potent than **8**. Also, ursane triterpenoids **13** and **14** are more potent than **44**. (3) A 9(11)-ene functionality shows similar potency to the original 12-ene (compare **18** with **8**). (4) The saturated ring C, 11,-13(18)-diene, and 9,11-epoxide are less potent than the original 12-ene (compare **19**–**21** with **8**). (5) Carboxyl, methoxycarbonyl, and nitrile groups at C-2 enhance potency.[5] Oleanane triterpenoids **3**–**7** (IC_{50} = 0.01–0.1 μM level) are about 10–100 times more potent than **8**. Ursane triterpenoids **38** and **39** are more potent than **44**. (6) The combination of a 9(11)-en-12-one functionality with nitrile and carboxyl groups at C-2 enhances the potency synergistically. Oleanane triterpenoids **25**, CDDO (**26**), and **29** (IC_{50} = 0.1 nM level) are about 10 000 times more potent than **8** (see Figure 1). (7) Although compounds **27** and **30** were also expected to show similar

Table 1. Activity of Olean-1-en-3-one and Urs-1-en-3-one Triterpenoids



olean-1-en-3-one



urs-1-en-3-one

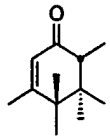
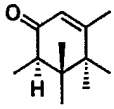
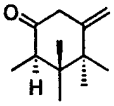
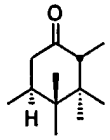
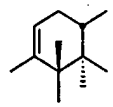
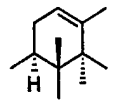
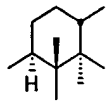
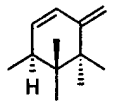
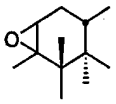
compd	skeleton[a]	structure of ring C	R_1 at C-2	R_2 at C-17	formula	analyses[b]	activity[c] IC_{50} (μM)
9	O		H	CO_2Me	$C_{31}H_{44}O_4 \cdot 1/3H_2O$	C,H	0.7
10	O		H	CO_2H	$C_{30}H_{42}O_4 \cdot 1/3H_2O$	C,H	0.2
25	O		CN	CO_2Me	$C_{32}H_{43}O_4N$	C,H,N	0.0001
26	O		CN	CO_2H	$C_{31}H_{41}O_4N$	C,H,N	0.0002
27	O		CO_2Me	CO_2Me	$C_{33}H_{46}O_6$	C,H	toxic[d]
28	O		CO_2Me	CO_2H	$C_{32}H_{44}O_6 \cdot 1/3H_2O$	C,H	0.1
29	O		CO_2H	CO_2Me	$C_{32}H_{44}O_6 \cdot 1/2H_2O$	C,H	0.0008
30	O		CO_2H	CO_2H	$C_{31}H_{42}O_6 \cdot 1/2H_2O$	C,H	0.2
36	O		$CONH_2$	CO_2Me	$C_{32}H_{45}O_5N \cdot 1/3H_2O$	C,H,N	0.1
37	O		CHO	CO_2Me	$C_{32}H_{44}O_5 \cdot 5/4H_2O$	C,H	0.1
11	O		H	CO_2Me	$C_{31}H_{44}O_4$	C,H	2.8
12	O		H	CO_2H	$C_{30}H_{42}O_4 \cdot 1/4H_2O$	C,H	1.1
13	U		H	CO_2Me	$C_{31}H_{44}O_4 \cdot 1/4H_2O$	C,H	8.9
14	U		H	CO_2H	$C_{30}H_{42}O_4 \cdot 1/4H_2O$	C,H	5.1
22	O		H	CH_2OAc	$C_{32}H_{46}O_4$	C,H	>40
23	O		H	CH_2OH	$C_{30}H_{44}O_3 \cdot 1/2H_2O$	C,H	3.0
24	O		H	CHO	$C_{30}H_{42}O_3 \cdot 1/2H_2O$	C,H	3.8
31	O		CN	CO_2Me	$C_{32}H_{43}O_4N \cdot 1/3H_2O$	[e]C,H,N	0.02
32	O		CN	CO_2H	$C_{31}H_{41}O_4N \cdot 1/3H_2O$	C,H,N	0.04
33	U		CN	CO_2Me	$C_{32}H_{43}O_4N$	C,H,N	0.1
34	U		CN	CO_2H	$C_{31}H_{41}O_4N \cdot H_2O$	C,H,N	0.8
15	O		H	CO_2H	$C_{30}H_{42}O_4 \cdot 3/4H_2O$	C,H	2.6
35	O		CN	CO_2H	$C_{31}H_{41}O_4N \cdot 1/2H_2O$	C,H,N	0.07
16	O		H	CO_2Me	$C_{31}H_{46}O_4$	C,H	14
17	O		H	CO_2H	$C_{30}H_{44}O_4 \cdot 2/3H_2O$	C,H	3.3
18	O		H	CO_2H	$C_{30}H_{44}O_3 \cdot 1/2H_2O$	C,H	5.2
43	O		H	CO_2Me	$C_{31}H_{46}O_3$	ref 32	31
8	O		H	CO_2H	$C_{30}H_{44}O_3 \cdot 3/4H_2O$	ref 5	5.6
44	U		H	CO_2H	$C_{30}H_{44}O_3$	ref 33	13
3	O		CN	CO_2Me	$C_{32}H_{45}O_3N \cdot 1/4H_2O$	ref 5	0.7
4	O		CN	CO_2H	$C_{31}H_{43}O_3N \cdot 1/2H_2O$	ref 5	0.6
38	U		CN	CO_2Me	$C_{32}H_{45}O_3N \cdot 3/4H_2O$	ref 5	5.1
39	U		CN	CO_2H	$C_{31}H_{43}O_3N \cdot H_2O$	ref 5	6.2
5	O		CO_2Me	CO_2Me	$C_{33}H_{48}O_5$	ref 5	0.9
40	O		CO_2Me	CO_2H	$C_{32}H_{46}O_5$	ref 5	2.2
6	O		CO_2H	CO_2Me	$C_{32}H_{46}O_5 \cdot 1/2H_2O$	ref 5	0.8
7	O		CO_2H	CO_2H	$C_{31}H_{44}O_5$	ref 5	0.07
41	O		$CONH_2$	CO_2Me	$C_{32}H_{47}O_4N \cdot 3/4H_2O$	ref 5	14
42	O		CHO	CO_2Me	$C_{32}H_{46}O_4$	ref 5	toxic[d]

Table 1 (Continued)

compd	skeleton[a]	structure of ring C	R_1 at C-2	R_2 at C-17	formula	analyses[b]	activity[c] IC_{50}(μM)
19	O		H	CO_2H	$C_{30}H_{46}O_3 \cdot 2/3H_2O$	C,H	8.5
20	O		H	CO_2H	$C_{30}H_{42}O_3 \cdot H_2O$	C,H	9.7
21	O		H	CO_2H	$C_{30}H_{44}O_4 \cdot 1/2H_2O$	C,H	36
1	oleanolic acid						>40
2	ursolic acid						toxic[e]
	hydrocortisone						0.01
	dexamethasone						0.0001

[a] O, olean-1-en-3-one; U, urs-1-en-3-one. [b] C, H, and N analyses were within ±0.4% of the theoretical values. [c] Details of the evaluation method are described in the Experimental Section. IC_{50} values of compounds **7**, **25**, **26**, **29**, **31**, **32**, **35**, hydrocortisone, and dexamethasone were determined in the range of 0.1 pM−1 μM (10-fold dilutions). The other compounds were assayed in the range of 0.01−40 μM (4-fold dilutions). Values are an average of two separate experiments. [d] Compounds **27** and **42** were toxic to cells above 1 μM and were not active below 1 μM. [e] Ursolic acid (**2**) was toxic to cells above 10 μM and was not active below 10 μM.

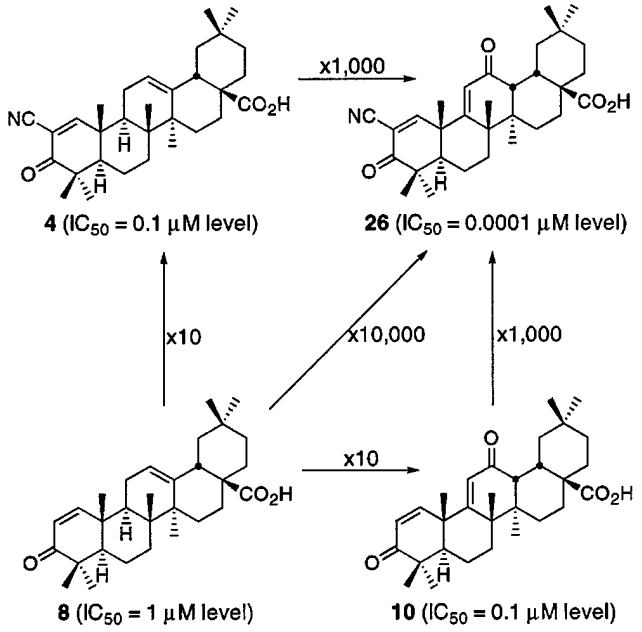


Figure 1. SARs between CDDO (**26**) and its lead compounds **4**, **8**, and **10**.

high potency to CDDO from the perspective of SARs, they did not (compare them with **5** and **7**). The reason diacid **30** did not show high potency might be that the higher polarity than that of monoacids **26** and **29** influences the bioavailability and permeability toward macrophages. (8) The combination of a 9(11)-en-12-one functionality with amide and formyl groups at C-2 does not enhance potency as strongly as a nitrile or carboxyl group as expected from the consideration of the activity of oleana-1,12-dien-3-one with amide and formyl groups at C-2 (compare **36** and **37** with **41** and **42**, respectively). (9) The combination of 12-en-11-one and 13(18)-en-11-one functionalities with a nitrile group at C-2 also strongly enhances the potency. Oleanane triterpenoids **31**, **32**, and **35** (IC_{50} = 0.01 μM level) are about 100 times more potent than **8**. Also, ursane triterpenoids **33** and **34** (IC_{50} = 0.1 μM level) are about 100 times more potent than **44** (IC_{50} = 10 μM level). (10) The oleanane skeleton is more potent than the ursane skeleton. Oleanane derivatives **3**, **4**, **8**, **11**, **12**, **31**, and **32** are more potent than ursane derivatives **38**, **39**, **44**, **13**, **14**, **33**, and **34**, respectively. (11) Acetoxymethyl, hydroxymethyl, and formyl groups at C-17 decrease potency compared with the carboxyl group at C-17 (compare **22**−**24** with **12**). (12) The role of methoxycarbonyl and carboxyl groups at C-17 is ambiguous. In some analogues, the carboxyl group is more potent than the methoxycarbonyl group: acids **7**, **8**, **17**, and **28** are more potent than esters **6**, **43**, **16**, and **27**, respectively. For other analogues, the carboxyl and methoxycarbonyl groups show similar potency: acids **4**, **26**, **32**, and **39** show similar potency to esters **3**, **25**, **31**, and **38**, respectively. Acids and esters with a nitrile group at C-2 seem to show this tendency although the reason is unknown. Lastly, acids **30** and **40** are less potent than esters **29** and **5**, respectively.

The selected oleanane triterpenoid, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) (**26**), was found to be a potent, multifunctional agent in various in vitro assays.[29] For example, CDDO induces monocytic differentiation of human myeloid leukemia cells and adipogenic differentiation of mouse 3T3-L1 fibroblasts.[30] CDDO inhibits proliferation of many human tumor cell lines. CDDO blocks de novo synthesis of inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. CDDO will protect rat brain hippocampal neurons from cell death induced by β-amyloid. The above potencies have been found at concentrations ranging from 10^{-6} to 10^{-9} M in cell culture. In addition, CDDO shows antiinflammatory activity against thioglycollate−IFN-γ-induced mouse peritonitis (0.1 μmol of CDDO/mouse, ip: a complete

suppression of both NO production and iNOS protein synthesis; 0.01 μmol of CDDO/mouse, ip: more than 50% suppression in these measurements).[31] CDDO may be a potential drug candidate for inflammatory diseases and chemoprevention of cancer.

Currently, further biological evaluation of CDDO, **25**, and **29** in vitro and in vivo for both antiinflammation and chemoprevention is in progress. Further studies on the mechanism of action of these compounds also are in progress.

Experimental Section

General Experimental Procedures. Melting points were determined on a Thomas-Hoover capillary melting point apparatus and are uncorrected. Optical rotations were measured with a Jasco DIP-181 digital polarimeter. UV and IR spectra were recorded on a Hewlett-Packard 8451A UV/VIS spectrophotometer and a Perkin-Elmer 600 series FTIR spectrophotometer, respectively. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Varian XL-300 Fourier transform spectrometer unless otherwise stated. The chemical shifts are reported in δ (ppm) using the δ 7.27 signal of C*H*Cl$_3$ (^{1}H NMR) and δ 77.23 signal of *C*DCl$_3$ (^{13}C NMR) as an internal standard unless otherwise stated. Low-resolution mass spectra and high-resolution MS data were obtained on a Micromass 70-VSE unless otherwise stated. Elemental microanalysis was performed by Atlantic Microlab Inc. TLC and preparative TLC (prep-TLC) were performed with Merck precoated TLC plates silica gel 60 F_{254}. Flash column chromatography was done with Select Scientific silica gel (230–400 mesh). The standard workup method was as follows: an organic extract was washed with saturated aqueous $NaHCO_3$ solution (three times) followed by saturated aqueous NaCl solution (three times), then dried over anhydrous $MgSO_4$, and filtered. The filtrate was evaporated in vacuo.

Methyl 3,12-Dioxooleana-1,9(11)-dien-28-oate (9). A solution of **54** (145 mg, 0.30 mmol) and phenylselenenyl chloride (98%) (69 mg, 0.35 mmol) in EtOAc (7 mL) was stirred at room temperature for 2.5 h. To the stirred mixture was added water (1.5 mL). After most of the aqueous layer was removed, THF (2.7 mL) and 30% H_2O_2 (0.24 mL) were added to the organic layer. The mixture was stirred at room temperature for 1 h. The mixture was worked up according to the standard method to give a crude solid (134 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (5:1)] to give **9** as an amorphous solid (96 mg, 67%): $[\alpha]^{23}_D$ +58° (*c* 0.64, CHCl$_3$). UV (EtOH) λ_{max} (log ϵ): 240 (4.20) nm. IR (KBr): 2948, 2872, 1723, 1666, 1598 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 7.33 (1H, d, J = 10.5 Hz), 6.00 (1H, s), 5.92 (1H, d, J = 10.5 Hz), 3.69 (3H, s), 3.04 (1H, ddd, J = 3.4, 4.6, 13.4 Hz), 2.91 (1H, d, J = 4.6 Hz), 1.41, 1.31, 1.19, 1.12, 1.01, 1.00, 0.89 (each 3H, s). ^{13}C NMR (CDCl$_3$): δ 203.7, 199.8, 178.4, 171.6, 155.0, 126.1, 123.8, 52.1, 49.8, 48.5, 47.4, 45.8, 44.9, 42.2, 42.0, 36.0, 34.7, 33.5, 33.0, 32.3, 31.7, 30.8, 28.2, 27.3, 27.1, 24.7, 23.3, 22.8, 21.84, 21.81, 18.6. EIMS (70 eV) *m/z*: 480 [M]$^+$ (99), 465 (100), 446 (42), 405 (27), 315 (41), 244 (44). HREIMS Calcd for $C_{31}H_{44}O_4$: 480.3240. Found: 480.3238. Anal. (Table 1).

3,12-Dioxooleana-1,9(11)-dien-28-oic Acid (10). A mixture of **9** (82 mg, 0.17 mmol) and LiI (405 mg) in dry DMF (2 mL) was heated under reflux for 7.5 h. To the mixture were added water and 5% aqueous HCl solution. The mixture was extracted with a mixture of CH_2Cl_2 and Et_2O (1:2) (three times). The extract was worked up according to the standard method to give an amorphous solid (78 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (1:1)] to give **10** as a crystalline solid (54 mg, 68%). An analytically pure sample was obtained by recrystallization from a mixture of hexanes and EtOAc (2:1) as colorless needles: mp >270 °C dec; $[\alpha]^{23}_D$ +63° (*c* 0.42, CHCl$_3$). UV (EtOH) λ_{max} (log ϵ): 240 (4.14) nm. IR (KBr): 3117, 2973, 2941, 2867, 1734, 1710, 1671, 1639, 1598 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 7.33 (1H, d, J = 10.6 Hz), 6.02 (1H, s), 5.93 (1H, d, J = 10.6 Hz), 3.02 (1H, ddd, J = 3.4, 4.9, 13.7 Hz), 2.96 (1H, d, J = 4.9 Hz), 1.41, 1.32, 1.19, 1.11, 1.02, 1.00, 0.90 (each 3H, s). ^{13}C NMR (CDCl$_3$): δ 203.8, 199.6, 183.9, 171.7, 155.0, 126.1, 123.8, 49.9, 48.4, 47.2, 45.8, 44.8, 42.2, 41.9, 35.9, 34.6, 33.4, 33.1, 32.3, 31.6, 30.8, 28.2, 27.3, 27.1, 24.8, 23.2, 22.7, 21.83, 21.75, 18.5. EIMS (70 eV) *m/z*: 466 [M]$^+$ (100), 451 (42), 301 (17), 244 (45). HREIMS Calcd for $C_{30}H_{42}O_4$: 466.3083. Found: 466.3064. Anal. (Table 1).

Methyl 3,11-Dioxooleana-1,12-dien-28-oate (11). 11 was prepared from methyl 3,11-dioxoolean-12-en-28-oate (**45**)[7] according to the same method as for **9** to give a crystalline solid (97%). This material was used for the next reaction without further purification. An analytically pure sample was obtained by flash column chromatography [hexanes–EtOAc (3:1)], followed by recrystallization from a mixture of hexanes and EtOAc (3:1) as crystals: mp 189–191 °C; $[\alpha]^{24}_D$ +152° (*c* 0.34, CHCl$_3$). UV (EtOH) λ_{max} (log ϵ): 248 (4.26) nm. IR (KBr): 2942, 2861, 1725, 1666, 1648 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 7.79 (1H, d, J = 10.3 Hz), 5.81 (1H, d, J = 10.3 Hz), 5.74 (1H, s), 3.66 (3H, s), 3.05 (1H, dd, J = 4.6, 14.9 Hz), 2.67 (1H, s), 2.08 (1H, ddd, J = 4.0, 13.7, 13.7 Hz), 1.39 (6H, s), 1.16, 1.11, 0.97, 0.96, 0.95 (each 3H, s). ^{13}C NMR (CDCl$_3$): δ 204.7, 199.3, 177.6, 170.4, 161.8, 127.6, 124.8, 55.7, 52.9, 52.1, 46.3, 45.3, 44.9, 44.4, 43.9, 42.0, 39.1, 33.8, 33.0, 32.3, 31.7, 30.9, 28.0, 27.8, 23.8, 23.6, 23.0, 21.7, 20.1, 19.4, 18.3. EIMS (70 eV) *m/z*: 480 [M]$^+$ (88), 465 (15), 421 (24), 397 (52), 276 (36), 257 (47), 217 (100). HREIMS Calcd for $C_{31}H_{44}O_4$: 480.3240. Found: 480.3231. Anal. (Table 1).

3,11-Dioxooleana-1,12-dien-28-oic Acid (12) and 3,11-Dioxooleana-1,13(18)-dien-28-oic Acid (15). 12 and **15** were prepared from **11** by the similar method as for **10** except that the reaction time was 2 h. The reaction mixture was subjected to prep-TLC [hexanes–EtOAc (3:5)] to give **12** as an amorphous solid (43%) and **15** as a crystalline solid (22%). **12**: $[\alpha]^{24}_D$ +161° (*c* 0.51, CHCl$_3$). UV (EtOH) λ_{max} (log ϵ): 248 (4.35) nm. IR (KBr): 3154, 2948, 2869, 1732, 1652, 1620 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 7.77 (1H, d, J = 10.3 Hz), 5.81 (1H, d, J = 10.3 Hz), 5.74 (1H, s), 3.02 (1H, dd, J = 4.3, 13.6 Hz), 2.67 (1H, s), 2.09 (1H, ddd, J = 5.2, 14.3, 14.3 Hz), 1.39, 1.38, 1.15, 1.08, 0.97, 0.96, 0.95 (each 3H, s). ^{13}C NMR (CDCl$_3$): δ 204.8, 199.4, 183.2, 170.1, 161.8, 127.9, 124.9, 55.7, 52.9, 46.2, 45.4, 44.9, 44.3, 44.0, 41.8, 39.1, 33.8, 33.0, 32.4, 31.7, 30.9, 28.0, 27.9, 23.8, 23.6, 22.7, 21.7, 20.2, 19.7, 18.2. FABMS (NBA, by a VG analytical ZAB 2SE) *m/z*: 467 [M + H]$^+$. HRFABMS (by a VG analytical ZAB 2SE) Calcd for $C_{30}H_{42}O_4$ + H: 467.3161. Found: 467.3161. Anal. (Table 1). **15**: mp >190 °C dec; $[\alpha]^{25}_D$ −16° (*c* 0.26, CHCl$_3$). UV (EtOH) λ_{max} (log ϵ): 210 (4.16), 226 (4.15), 300 (3.16) nm. IR (KBr): 3200, 2946, 2866, 1692 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 7.46 (1H, d, J = 10.1 Hz), 5.82 (1H, d, J = 10.1 Hz), 3.56 (1H, d, J = 17.8 Hz), 2.88 (1H, d, J = 17.8 Hz), 2.65 (1H, s), 2.31 (2H, m), 2.09 (1H, m), 1.44, 1.31, 1.16, 1.10, 0.96, 0.93, 0.76 (each 3H, s). ^{13}C NMR (CDCl$_3$): δ 208.6, 204.8, 181.9, 160.8, 133.8, 129.9, 125.1, 58.0, 52.9, 48.1, 44.8, 44.3, 44.1, 43.4, 41.1, 39.1, 36.7, 35.7, 33.03, 32.95, 32,8, 32.2, 27.7, 26.6, 24.2, 21.8, 20.2, 20.1, 19.2, 18.8. FABMS (NBA, by a VG analytical ZAB 2SE) *m/z*: 467 [M + H]$^+$. HRFABMS (by a VG analytical ZAB 2SE) Calcd for $C_{30}H_{42}O_4$ + H: 467.3161. Found: 467.3187. Anal. (Table 1).

Methyl 3,11-Dioxoursa-1,12-dien-28-oate (13). 13 was prepared from **48** according to the same method as for **9** to give a crystalline solid (93%). An analytically pure sample was obtained by recrystallization from a mixture of hexanes and EtOAc (3:1) as crystals: mp 172–174 °C; $[\alpha]^{24}_D$ +150° (*c* 0.49, CHCl$_3$). UV (EtOH) λ_{max} (log ϵ): 248 (4.26) nm. IR (KBr): 2973, 2948, 2866, 1726, 1670, 1655, 1610 cm^{-1}. ^{1}H NMR (CDCl$_3$): δ 7.75 (1H, d, J = 10.3 Hz), 5.82 (1H, d, J = 10.3 Hz), 5.71 (1H, s), 3.63 (3H, s), 2.64 (1H, s), 2.47 (1H, d, J = 11.7 Hz), 2.11 (1H, ddd, J = 4.6, 14.7, 14.7 Hz), 1.41, 1.33, 1.16, 1.11 (each 3H, s), 0.98 (3H, d, J = 7.2 Hz), 0.97 (3H, s), 0.89 (3H, d, J = 6.6 Hz). ^{13}C NMR (CDCl$_3$): δ 204.7, 198.8, 177.3, 164.5, 161.8, 130.4, 124.8, 55.5, 53.1, 53.0, 52.1, 47.9, 45.0, 44.9, 44.2, 39.0, 38.82, 38.79, 36.1, 32.5, 30.5, 28.7, 27.8, 24.0, 21.8, 21.3, 21.2, 20.1, 19.4, 18.3, 17.3. EIMS (70 eV) *m/z*: 480 [M]$^+$ (84), 465

(19), 421 (15), 397 (100), 257 (38), 217 (39). HREIMS Calcd for $C_{31}H_{44}O_4$: 480.3240. Found: 480.3239. Anal. (Table 1).

3,11-Dioxoursa-1,12-dien-28-oic Acid (14). 14 was prepared from **13** by the similar method as for **10** except that the reaction time was 1.25 h. The reaction mixture was crystallized from a mixture of hexanes and EtOAc (2:1) to give **14** as crystals (58%). An analytically pure sample was obtained by recrystallization from MeOH as colorless needles: mp >275 °C dec; $[\alpha]^{24}_D$ +157° (*c* 0.29, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 247 (4.17) nm. IR (KBr): 3116, 2983, 2950, 2930, 1720, 1668, 1628 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.74 (1H, d, J= 10.3 Hz), 5.82 (1H, d, J = 10.3 Hz), 5.71 (1H, s), 2.65 (1H, s), 2.44 (1H, d, J = 11.2 Hz), 2.13 (1H, m), 1.41, 1.34, 1.15, 1.08 (each 3H, s), 0.99 (3H, d, J= 7.2 Hz), 0.97 (3H, s), 0.89 (3H, d, J= 6.3 Hz). ^{13}C NMR ($CDCl_3$): δ 204.8, 199.0, 183.1, 164.3, 161.7, 130.6, 124.8, 55.4, 52.9, 52.8, 47.7, 45.0, 44.9, 44.2, 39.0, 38.8, 38.7, 36.2, 32.5, 30.4, 28.6, 27.8, 23.7, 21.7, 21.3, 21.1, 20.2, 19.6, 18.2, 17.2. FABMS (NBA, by a VG analytical ZAB 2SE) *m/z*: 467 $[M + H]^+$. HRFABMS (by a VG analytical ZAB 2SE) Calcd for $C_{30}H_{42}O_4$ + H: 467.3161. Found: 467.3202. Anal. (Table 1).

3,11-Dioxooleana-1,13(18)-dien-28-oic Acid (15). To a solution of **20** (106 mg, 0.24 mmol) in acetone (6.5 mL) was added Jones reagent (0.36 mL) dropwise in an ice bath. The mixture was stirred at room temperature for 30 min. After removal of acetone, water was added to the resultant mixture. The aqueous mixture was extracted with CH_2Cl_2 (three times). The extract was worked up according to the standard method to give a solid (80 mg). The solid was subjected to prep-TLC [hexanes–EtOAc (1.2:1.0)] to give **15** as a crystalline solid (31 mg, 28%).

Methyl 3,12-Dioxoolean-1-en-28-oate (16). 16 was prepared from **51** according to the same method as for **9**. The crude solid was subjected to flash column chromatography [hexanes–EtOAc (3:1) followed by hexanes–EtOAc (2:1)] to give **16** as an amorphous solid (75%): $[\alpha]^{24}_D$ +2.1° (*c* 0.39, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 234 (3.85) nm. IR (KBr): 2946, 2867, 1724, 1700, 1671 cm^{-1}. ^1H NMR ($CDCl_3$): δ 6.95 (1H, d, J= 10.3 Hz), 5.84 (1H, d, J= 10.3 Hz), 3.70 (3H, s), 2.82 (1H, ddd, J= 3.5, 4.2, 13.4 Hz), 2.68 (1H, d, J= 4.2 Hz), 2.49 (1H, dd, J= 4.6, 16.4 Hz), 2.33 (1H, dd, J= 13.3, 16.4 Hz), 1.16, 1.11, 1.10, 1.06, 0.99, 0.97, 0.91 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 210.4, 204.8, 178.5, 157.2, 126.0, 53.4, 52.2, 52.1, 47.5, 44.8, 44.2, 42.4, 42.3, 39.5, 38.6, 36.4, 34.6, 33.5, 33.0, 32.2, 31.6, 30.8, 27.82, 27.76, 23.3, 22.9, 21.6, 20.8, 19.1, 18.5, 16.6. EIMS (70 eV) *m/z*: 482 $[M]^+$ (5.5), 467 (42), 407 (100), 278 (25), 218 (64). HREIMS Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3387. Anal. (Table 1).

3,12-Dioxoolean-1-en-28-oic Acid (17). 17 was prepared from **16** by the similar method as for **10** except that the reaction time was 4.5 h. The crude material was subjected to prep-TLC [hexanes–EtOAc (1:2)] to give **17** as a crystalline solid (62%): mp 243–245 °C dec; $[\alpha]^{24}_D$ +2.3° (*c* 0.27, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 234 (3.89) nm. IR (KBr): 3166, 2946, 2866, 1722, 1696, 1668, 1651 cm^{-1}. ^1H NMR ($CDCl_3$): δ 6.96 (1H, d, J= 10.4 Hz), 5.84 (1H, d, J= 10.4 Hz), 2.79 (2H, m), 2.51 (1H, dd, J= 4.9, 15.9 Hz), 2.35 (1H, dd, J= 13.2, 15.9 Hz), 1.17, 1.11 (each 3H, s), 1.10 (6H, s), 1.00, 0.98, 0.93 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 210.2, 204.9, 184.2, 157.2, 126.0, 53.3, 52.2, 47.4, 44.8, 44.1, 42.4, 42.3, 39.5, 38.6, 36.2, 34.6, 33.5, 33.2, 32.0, 31.6, 30.8, 27.8, 23.3, 22.8, 21.6, 20.7, 19.1, 18.5, 16.7. EIMS (70 eV) *m/z*: 468 $[M]^+$ (9.7), 453 (15), 407 (39), 218 (19), 83 (100). HREIMS Calcd for $C_{30}H_{44}O_4$: 468.3240. Found: 468.3221. Anal. (Table 1).

3-Oxooleana-1,9(11)-dien-28-oic Acid (18). 18 was prepared from **58** according to the same method as for **9**. The crude solid was subjected to prep-TLC [hexanes–EtOAc (2:1)] to give **18** as a crystalline solid (80%). An analytically pure sample was obtained by recrystallization from MeOH as colorless needles: mp >240 °C dec; $[\alpha]^{24}_D$ +55° (*c* 0.28, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 234 (3.93) nm. IR (KBr): 3138, 3053, 2959, 2930, 2869, 1727, 1693, 1645 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.42 (1H, d, J= 10.4 Hz), 5.85 (1H, d, J= 10.4 Hz), 5.63 (1H, t, J= 3.4 Hz), 1.35, (3H, s), 1.16 (6H, s), 1.07, 0.94 (each 3H, s), 0.90 (6H, s). ^{13}C NMR ($CDCl_3$): δ 205.0, 185.0, 157.9, 147.7, 124.4, 118.6, 49.9, 48.1, 44.7, 44.1, 41.3, 38.6, 36.2, 35.6, 34.4, 33.7, 33.6, 33.2, 31.8, 30.8, 28.5, 28.0, 27.2, 26.9, 26.3, 23.6, 23.4, 21.7, 18.8, 18.7. FABMS (NBA, by a VG analytical ZAB 2SE) *m/z*: 453 $[M + H]^+$. HRFABMS (by a VG analytical ZAB 2SE) Calcd for $C_{30}H_{44}O_3$ + H: 453.3369. Found: 453.3390. Anal. (Table 1).

3-Oxoolean-1-en-28-oic Acid (19). 19 was prepared from 3-oxoolean-28-oic acid (**56**)[15] according to the same method as for **9**. The crude solid was subjected to flash column chromatography [hexanes–EtOAc (3:1)] to give **19** as an amorphous solid (68%): $[\alpha]^{24}_D$ +30° (*c* 0.55, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 236 (3.90) nm. IR (KBr): 3200, 2944, 2866, 1729, 1692, 1672 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.11 (1H, d, J= 10.2 Hz), 5.82 (1H, d, J= 10.2 Hz), 2.22 (1H, m), 1.13, 1.06, 1.04, 0.99, 0.96, 0.92, 0.88 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 205.7, 184.9, 159.8, 125.4, 53.5, 48.1, 44.8, 44.7, 42.9, 40.8, 39.7, 37.4, 36.7, 36.5, 34.5, 33.6, 33.4, 32.5, 30.6, 28.5, 28.0, 26.9, 23.6, 23.3, 21.6, 19.2, 17.2, 16.9. FABMS (NBA, by a Micromass ZAB-SE) *m/z*: 455 $[M + H]^+$. HRFABMS (by a Micromass 70-SE-4F) Calcd for $C_{30}H_{46}O_3$ + H: 455.3525. Found: 455.3518. Anal. (Table 1).

3-Oxooleana-1,11,13(18)-trien-28-oic Acid (20). 20 was prepared from **60** by the similar method as for **10** except that the reaction time was 4 h. The crude solid was subjected to prep-TLC [hexanes–EtOAc (2.5:1)] to give **20** as an amorphous solid (56%): $[\alpha]^{24}_D$ −88° (*c* 0.44, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 246 (4.35), 252 (4.35) nm. IR (KBr): 3167, 3036, 2944, 2863, 1727, 1695, 1672 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.27 (1H, d, J= 10.1 Hz), 6.57 (1H, dd, J= 2.9, 10.5 Hz), 5.89 (1H, d, J= 10.1 Hz), 5.81 (1H, dd, J= 1.5, 10.5 Hz), 2.57 (1H, d, J= 14.2 Hz), 2.29 (2H, m), 1.174, 1.170, 1.10, 1.00, 0.98, 0.86, 0.82 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 205.7, 182.8, 159.1, 136.6, 132.5, 126.5, 125.7, 125.4, 53.4, 48.4, 48.3, 45.0, 42.4, 41.6, 40.8, 39.3, 37.0, 35.6, 32.9, 32.7, 32.4, 31.9, 27.7, 25.1, 24.3, 21.32, 21.27, 20.0, 19.2, 16.9. FABMS (NBA, by a VG analytical ZAB 2SE) *m/z*: 451 $[M + H]^+$. HRFABMS (by a VG analytical ZAB 2SE) Calcd for $C_{30}H_{42}O_3$ + H: 451.3212. Found: 451.3240. Anal. (Table 1).

9,11-Epoxy-3-oxoolean-1-en-28-oic Acid (21). A mixture of **18** (57 mg, 0.13 mmol) and *m*CPBA (60%) (43 mg, 0.15 mmol) in CH_2Cl_2 (3 mL) was stirred at room temperature overnight. After the mixture was diluted with a mixture of CH_2Cl_2 and Et_2O (1:2), it was worked up according to the standard method to give a solid (65 mg). The solid was subjected to prep-TLC [hexanes–EtOAc (1.5:1)] to give **21** as a crystalline solid (27 mg, 46%). An analytically pure sample was obtained by recrystallization from MeOH as colorless needles: mp 253–254 °C; $[\alpha]^{24}_D$ −14° (*c* 0.25, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 236 (3.88) nm. IR (KBr): 2970, 2945, 1688 cm^{-1}. ^1H NMR ($CDCl_3$): δ 6.55 (1H, d, J= 10.4 Hz), 5.85 (1H, d, J= 10.4 Hz), 3.02 (1H, s), 1.39 (3H, s), 1.07 (6H, s), 1.04, 0.96, 0.92, 0.87 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 204.5, 184.4, 154.4, 125.2, 67.8, 60.2, 47.9, 45.3, 44.9, 42.3, 41.5, 38.4, 37.3, 35.7, 34.3, 33.6, 33.3, 30.8, 30.0, 28.2, 27.9, 26.9, 24.9, 23.6, 23.3, 21.1, 20.6, 18.7, 18.6. FABMS (NBA, by a VG analytical ZAB 2SE) *m/z*: 469 $[M + H]^+$. HRFABMS (by a VG analytical ZAB 2SE) Calcd for $C_{30}H_{44}O_4$ + H: 469.3318. Found: 469.3314. Anal. (Table 1).

3,11-Dioxooleana-1,12-dien-28-yl Acetate (22). 22 was prepared from 3,11-dioxoolean-12-en-28-yl acetate (**61**)[18] according to the same method as for **9**. The crude solid was subjected to prep-TLC [hexanes–EtOAc (3:1)] to give **22** as an amorphous solid (83%): $[\alpha]^{24}_D$ +131° (*c* 0.45, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 246 (4.31) nm. IR (KBr): 2949, 2868, 1742, 1665 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.72 (1H, d, J= 10.1 Hz), 5.79 (1H, d, J= 10.1 Hz), 5.67 (1H, s), 3.97 (1H, d, J= 11.2 Hz), 3.71 (1H, d, J= 11.2 Hz), 2.66 (1H, s), 2.29 (1H, dd, J= 4.2, 13.2 Hz), 2.07 (3H, s), 2.03 (1H, ddd, J= 4.4, 13.9, 13.9 Hz), 1.404, 1.397, 1.18, 1.15, 1.11, 0.93, 0.91 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 204.7, 198.9, 171.2, 170.2, 161.7, 128.3, 124.8, 70.3, 55.8, 53.0, 45.7, 44.99, 44.96, 43.8, 42.9, 39.0, 36.0, 33.9, 33.0, 32.1, 31.2, 31.0, 27.8, 26.1, 23.7, 23.5, 22.1, 21.7, 21.1, 20.2, 19.0, 18.3. EIMS (70 eV) *m/z*: 494 $[M]^+$ (100), 446 (92), 411

(41), 406 (37), 351 (19). HREIMS Calcd for $C_{32}H_{46}O_4$: 494.3396. Found: 494.3396. Anal. (Table 1).

28-Hydroxyoleana-1,12-diene-3,11-dione (23). A solution of **22** (47 mg, 0.095 mmol) and KOH (300 mg) in MeOH (3 mL) was stirred at room temperature for 20 min. The mixture was acidified with 5% aqueous HCl solution. The aqueous mixture was extracted with a mixture of CH_2Cl_2 and Et_2O (1:2) (three times). The extract was worked up according to the standard method to give an amorphous solid (42 mg). The solid was subjected to prep-TLC [hexanes–EtOAc (1.7:1)] to give **23** as an amorphous solid (34 mg, 78%): $[\alpha]^{24}_D$ +145° (*c* 0.50, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 250 (4.13) nm. IR (KBr): 3477, 2947, 2865, 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.72 (1H, d, J = 10.3 Hz), 5.80 (1H, d, J = 10.3 Hz), 5.67 (1H, s), 3.48 (1H, d, J = 11.0 Hz), 3.25 (1H, d, J = 11.0 Hz), 2.67 (1H, s), 2.21 (1H, dd, J = 3.8, 13.6 Hz), 1.97 (1H, ddd, J = 4.4, 13.7, 13.7 Hz), 1.41, 1.40 (each 3H, s), 1.16 (6H, s), 1.11, 0.93, 0.91 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 204.8, 199.1, 171.4, 161.8, 128.0, 124.8, 69.8, 55.7, 53.0, 45.8, 45.1, 45.0, 43.9, 43.0, 39.0, 37.2, 34.0, 33.1, 32.2, 31.3, 30.8, 27.8, 26.1, 23.6, 21.8, 21.7, 20.3, 19.0, 18.4. EIMS (70 eV) *m/z*: 452 $[M]^+$ (100), 437 (15), 434 (16), 383 (16), 364 (50), 248 (46). HREIMS Calcd for $C_{30}H_{44}O_3$: 452.3290. Found: 452.3292. Anal. (Table 1).

Oleana-1,12-diene-3,11,28-trione (24). To a stirred mixture of CrO_3 (70 mg, 0.70 mmol) and pyridine (110 mg, 1.39 mmol) in dry CH_2Cl_2 (2 mL) was added a solution of **23** (53 mg, 0.12 mmol) in dry CH_2Cl_2 (1.5 mL). The mixture was stirred at room temperature for 15 min. The mixture was worked up according to Ratcliffe's procedure[20] to give a crude solid of **24** (47 mg, 89%). The solid was recrystallized from a mixture of hexanes and EtOAc (2:1) to give **24** as colorless needles (31 mg, 59%): mp >267 °C dec; $[\alpha]^{24}_D$ +160° (*c* 0.27, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 248 (4.15) nm. IR (KBr): 2944, 2864, 1719, 1674, 1644 cm^{-1}. ^1H NMR ($CDCl_3$): δ 9.40 (1H, s), 7.76 (1H, d, J = 10.3 Hz), 5.80 (1H, d, J = 10.3 Hz), 5.77 (1H, s), 2.84 (1H, dd, J = 4.3, 13.6 Hz), 2.64 (1H, s), 2.10 (1H, ddd, J = 3.9, 14.3, 14.3 Hz), 1.38 (6H, s), 1.15, 1.10 (each 3H, s), 0.96 (6H, s), 0.93 (3H, s). ^{13}C NMR ($CDCl_3$): δ 205.4, 204.7, 199.0, 169.1, 161.7, 128.0, 124.8, 55.7, 53.0, 49.1, 45.4, 44.9, 44.3, 43.9, 40.1, 39.1, 33.2, 32.9, 32.4, 30.9, 27.9, 27.30, 27.26, 23.5, 23.3, 21.7, 21.6, 20.1, 19.6, 18.3. EIMS (70 eV) *m/z*: 450 $[M]^+$ (100), 446 (64), 367 (45), 362 (31), 246 (36). HREIMS Calcd for $C_{30}H_{42}O_3$: 450.3134. Found: 450.3129. Anal. (Table 1).

Methyl 2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oate (25). A mixture of **64** (1.51 g, 2.97 mmol) and DDQ (98%) (0.77 g, 3.32 mmol) in dry benzene (80 mL) was heated under reflux for 30 min. After insoluble matter was removed by filtration, the filtrate was evaporated in vacuo to give a solid. The solid was subjected to flash column chromatography [benzene–acetone (10:1)] to give **25** as an amorphous solid (1.38 g, 92%): $[\alpha]^{23}_D$ +33° (*c* 0.68, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 244 (4.07) nm. IR (KBr): 2950, 2872, 2233, 1722, 1690, 1665 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.04 (1H, s), 5.96 (1H, s), 3.68 (3H, s), 3.02 (1H, ddd, J = 3.4, 4.9, 13.4 Hz), 2.92 (1H, d, J = 4.9 Hz), 1.47, 1.31, 1.24, 1.15, 0.99, 0.98, 0.88 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 199.0, 196.8, 178.3, 168.6, 165.9, 124.2, 114.7, 114.6, 52.1, 49.8, 47.8, 47.3, 45.9, 45.2, 42.7, 42.2, 35.9, 34.6, 33.4, 32.9, 31.8, 31.6, 30.8, 28.1, 27.1, 26.8, 24.7, 23.2, 22.7, 21.8, 21.7, 18.4. EIMS (70 eV) *m/z*: 505 $[M]^+$ (100), 490 (81), 430 (42), 315 (47), 269 (40). HREIMS Calcd for $C_{32}H_{43}O_4N$: 505.3192. Found: 505.3187. Anal. (Table 1).

2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-oic Acid (26). A mixture of **25** (612 mg, 1.21 mmol) and LiI (3.0 g) in dry DMF (10 mL) was heated under reflux for 4 h. To the mixture were added water and 5% aqueous HCl solution. The mixture was extracted with EtOAc (three times). The extract was washed with water (three times) and saturated aqueous NaCl solution (three times), dried over $MgSO_4$, and filtered. The filtrate was evaporated in vacuo to give an amorphous solid. The solid was subjected to flash column chromatography [hexanes–EtOAc (1:1) followed by CH_2Cl_2–MeOH (15:1)] to give crude **26** (530 mg). The crude product was purified by recrystallization from benzene to give crystals. To remove benzene completely, the crystals were dissolved in CH_2Cl_2 (20 mL) and the solvent was evaporated in vacuo to give benzene-free **26** as an amorphous solid (405 mg, 68%): $[\alpha]^{22}_D$ +33 ° (*c* 0.28, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 240 (4.21) nm. IR (KBr): 2950, 2867, 2235, 1692, 1665 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.05 (1H, s), 6.00 (1H, s), 3.06–2.98 (2H, m), 1.48, 1.34, 1.25, 1.16, 1.02, 1.00, 0.90 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 199.0, 196.8, 183.7, 168.8, 165.9, 124.2, 114.7, 114.5, 49.8, 47.8, 47.1, 45.9, 45.2, 42.7, 42.3, 35.8, 34.5, 33.3, 33.0, 31.8, 31.5, 30.8, 28.1, 27.1, 26.8, 24.8, 23.2, 22.6, 21.72, 21.71, 18.4. EIMS (70 eV) *m/z*: 491 $[M]^+$ (100), 476 (62), 445 (29), 430 (27), 269 (94). HREIMS Calcd for $C_{31}H_{41}O_4N$: 491.3036. Found: 491.3020. Anal. (Table 1).

Methyl 2-Methoxycarbonyl-3,12-dioxooleana-1,9(11)-dien-28-oate (27). To a solution of phenylselenenyl chloride (98%) (78 mg, 0.40 mmol) in CH_2Cl_2 (3.2 mL) in an ice bath was added a solution of pyridine (35 mg, 0.44 mmol) in CH_2Cl_2 (0.8 mL). After 15 min, a solution of **71** (108 mg, 0.20 mmol) in CH_2Cl_2 (1.4 mL) was added and the mixture was stirred an additional 1 h. After the mixture was washed with 10% aqueous HCl solution (1.6 mL) twice, 30% H_2O_2 (0.2 mL) was added to the stirred mixture in the ice bath. After an additional 40 min, the mixture was worked up according to the standard method to give a solid (108 mg). The solid was subjected to flash column chromatography [hexanes–EtOAc (2:1)] to afford **71** (21 mg) and **27** as colorless needles (76 mg; 71%, 88% based on recovered **71**): mp 187–188 °C; $[\alpha]^{23}_D$ +35° (*c* 0.38, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 246 (4.06) nm. IR (KBr): 2944, 2867, 1722, 1664, 1597 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.05 (1H, s), 6.09 (1H, s), 3.79, 3.69 (each 3H, s), 3.04 (1H, ddd, J = 3.5, 4.5, 13.9 Hz), 2.94 (1H, d, J = 4.5 Hz), 1.37, 1.30, 1.18, 1.17, 1.01, 0.99, 0.88 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 199.6, 199.4, 178.3, 170.8, 165.0, 160.7, 129.9, 125.2, 52.5, 52.1, 50.0, 48.3, 47.4, 46.0, 45.8, 42.3, 42.0, 36.0, 34.6, 33.4, 32.9, 31.7, 30.8, 28.2, 28.1, 27.3, 24.6, 23.3, 22.8, 21.7, 21.4, 18.8. EIMS (70 eV) *m/z*: 538 $[M]^+$ (20), 523 (40), 506 (100), 315 (47). HREIMS Calcd for $C_{33}H_{46}O_6$: 538.3294. Found: 538.3289. Anal. (Table 1).

2-Methoxycarbonyl-3,12-dioxooleana-1,9(11)-dien-28-oic Acid (28). A solution of **30** (33 mg, 0.064 mmol) in MeOH (3.1 mL) containing concentrated H_2SO_4 (0.09 mL) was heated under reflux for 25 min. After water was added to the mixture, it was extracted with EtOAc (three times). The extract was worked up according to the standard method to give a solid (31 mg). The solid was a mixture of **28** and 3-hydroxy-1-methoxy-2-methoxycarbonyl-12-oxooleana-2,9(11)-dien-28-oic acid (**72**). The solid was subjected to prep-TLC [hexanes–EtOAc (1:1)] to give only **28** as a crystalline solid (27 mg, 82%). An analytically pure sample was obtained by recrystallization from a mixture of hexanes and EtOAc (2:1) as colorless needles: mp >265 °C dec; $[\alpha]^{23}_D$ +34° (*c* 0.42, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 240 (4.09) nm. IR (KBr): 3118, 2977, 2940, 2869, 1718, 1692, 1636 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.06 (1H, s), 6.11 (1H, s), 3.81 (3H, s), 3.09–2.98 (2H, m), 1.38, 1.34, 1.20, 1.19, 1.04, 1.02, 0.91 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 199.7, 199.2, 183.5, 170.9, 165.1, 160.7, 130.0, 125.3, 52.6, 50.0, 48.3, 47.2, 46.0, 45.9, 42.3, 42.0, 35.9, 34.6, 33.4, 33.1, 31.7, 31.6, 30.8, 28.2, 28.1, 27.3, 24.7, 23.2, 22.7, 21.7, 21.4, 18.8. EIMS (70 eV) *m/z*: 524 $[M]^+$ (17), 509 (24), 492 (100), 446 (38), 302 (31). HREIMS Calcd for $C_{32}H_{44}O_6$: 524.3138. Found: 524.3142. Anal. (Table 1). **72**:[34] ^1H NMR ($CDCl_3$): δ 13.06 (1H, s), 5.93 (1H, s), 4.46 (1H, s), 3.82 (3H, s), 3.21 (3H, s), 3.03 (2H, m), 2.12 (1H, dd, J = 3.8, 10.4 Hz), 1.26, 1.22, 1.13, 1.07, 1.05, 1.02, 0.92 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 200.2, 184.1, 182.1, 174.8, 174.0, 124.4, 96.9, 57.3, 51.9, 50.1, 47.4, 46.0, 45.7, 44.7, 42.8, 41.5, 39.5, 36.1, 34.7, 33.4, 33.2, 31.7, 31.2, 30.9, 28.5, 24.3, 23.8, 23.3, 23.2, 22.8, 21.2, 20.9, 18.5. EIMS (70 eV) *m/z*: 556 $[M]^+$ (3.0), 538 (54), 524 (61), 509 (35), 492 (96), 446 (86), 315 (100). HREIMS Calcd for $C_{33}H_{48}O_7$: 556.3400. Found: 556.3410.

Methyl 2-Carboxy-3,12-dioxooleana-1,9(11)-dien-28-oate (29). A mixture of **27** (273 mg, 0.51 mmol) and KOH (1.6 g) in water (5.3 mL) and MeOH (16 mL) was heated under reflux for 15 min. After the mixture was acidified with 10% aqueous HCl solution, it was extracted with EtOAc (three

times). The extract was washed with water (three times) and saturated aqueous NaCl solution (three times), dried over $MgSO_4$, and filtered. The filtrate was evaporated in vacuo to give a solid (264 mg). The solid was recrystallized from MeOH to afford **29** as colorless needles (174 mg). The solid (75 mg) which was obtained from the mother liquid was subjected to flash column chromatography [hexanes−EtOAc (1:1)] to give **9** (19 mg, 8%) and **29** as colorless needles (33 mg, total 78%): mp 155−156 °C dec; $[\alpha]^{23}_D$ +50° (*c* 0.30, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 254 (4.14) nm. IR (KBr): 2950, 2872, 1756, 1722, 1664 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.77 (1H, s), 6.17 (1H, s), 3.70 (3H, s), 3.04 (1H, ddd, J = 3.6, 4.5, 13.2 Hz), 2.92 (1H, d, J = 4.5 Hz), 1.48, 1.34, 1.29, 1.22 (each 3H, s), 1.00 (6H, s), 0.90 (3H, s). ^{13}C NMR ($CDCl_3$): δ 207.6, 199.1, 178.4, 169.1, 168.5, 164.3, 124.5, 123.8, 52.1, 49.9, 47.7, 47.4, 45.9, 45.7, 42.5, 42.2, 35.9, 34.6, 33.4, 32.9, 31.8, 31.7, 30.8, 28.2, 27.5, 27.1, 24.8, 23.2, 22.8, 22.0, 21.8, 18.5. EIMS (70 eV) *m/z*: 524 $[M]^+$ (12), 509 (31), 506 (74), 480 (52), 465 (83), 405 (56), 315 (66), 175 (100). HREIMS Calcd for $C_{32}H_{44}O_6$: 524.3138. Found: 524.3138. Anal. (Table 1).

2-Carboxy-3,12-dioxooleana-1,9(11)-dien-28-oic Acid (30).[35] A mixture of **29** (120 mg, 0.23 mmol) and LiI (545 mg) in dry DMF (1.6 mL) was heated under reflux for 30 min. The reaction mixture was worked up according to the same method as for **26** to give a solid (125 mg). The solid was recrystallized from a mixture of hexanes and EtOAc (1:2) to afford **30** as colorless needles (36 mg). The solid which was obtained from the mother liquid was subjected to flash column chromatography [hexanes−EtOAc (1:2)] to give **10** (26 mg, 24%) and **30** as colorless needles (19 mg, total 47%): mp >260 °C dec; $[\alpha]^{24}_D$ +52° (*c* 0.28, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 256 (4.17) nm. IR (KBr): 3269, 2956, 2928, 1750, 1728, 1658, 1631, 1595 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.77 (1H, s), 6.18 (1H, s), 3.04 (1H, ddd, J = 3.5, 4.9, 13.6 Hz), 2.98 (1H, d, J = 4.9 Hz), 1.48, 1.36, 1.30, 1.23 (each 3H, s), 1.02 (6H, s), 0.91 (3H, s). EIMS (70 eV) *m/z*: 510 $[M]^+$ (12), 492 (100), 466 (71), 451 (75), 405 (48), 301 (37). HREIMS Calcd for $C_{31}H_{42}O_6$: 510.2981. Found: 510.2979. Anal. (Table 1).

Methyl 2-Cyano-3,11-dioxooleana-1,12-dien-28-oate (31). **31** was prepared from **67** according to the same method as for **25** to give an amorphous solid (80%): $[\alpha]^{24}_D$ +97° (*c* 0.49, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 250 (4.24) nm. IR (KBr): 2944, 2867, 2233, 1726, 1686, 1656, 1617 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.59 (1H, s), 5.77 (1H, s), 3.65 (3H, s), 3.06 (1H, dd, J = 4.0, 13.7 Hz), 2.69 (1H, s), 2.08 (1H, ddd, J = 4.1, 13.6, 13.6 Hz), 1.41, 1.38, 1.21, 1.15, 0.97, 0.96, 0.95 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 198.4, 197.8, 177.5, 173.0, 171.5, 127.3, 115.1, 113.5, 54.5, 52.2, 52.0, 46.3, 45.5, 45.3, 44.4, 44.1, 42.1, 40.0, 33.8, 33.0, 31.9, 31.6, 30.9, 28.0, 27.8, 23.8, 23.6, 23.0, 21.7, 19.6, 19.4, 18.2. EIMS (70 eV) *m/z*: 505 $[M]^+$ (100), 445 (22), 417 (27), 370 (20). HREIMS Calcd for $C_{32}H_{43}O_4N$: 505.3192. Found: 505.3200. Anal. (Table 1).

2-Cyano-3,11-dioxooleana-1,12-dien-28-oic Acid (32)[35] and 2-Cyano-3,11-dioxooleana-1,13(18)-dien-28-oic Acid (35). **32** and **35** were prepared from **31** by the similar method as for **26**. The reaction mixture was subjected to prep-TLC [hexanes−EtOAc−MeOH (50:100:1.5)] to give **32** as a crystalline solid (37%) and **35** as an amorphous solid (16%). **32**: mp >270 °C dec; $[\alpha]^{24}_D$ +101° (*c* 0.28, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 250 (4.23) nm. IR (KBr): 3228, 2944, 2867, 2233, 1732, 1689, 1656 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.58 (1H, s), 5.78 (1H, s), 3.04 (1H, dd, J = 3.7, 13.9 Hz), 2.69 (1H, s), 2.11 (1H, ddd, J = 3.9, 13.7, 13.7 Hz), 1.42, 1.40, 1.22, 1.14, 1.00, 0.973, 0.968 (each 3H, s). EIMS (70 eV) *m/z*: 491 $[M]^+$ (34), 445 (31), 397 (26), 257 (36), 189 (59), 95 (100). HREIMS Calcd for $C_{31}H_{41}O_4N$: 491.3036. Found: 491.3034. Anal. (Table 1). **35**: $[\alpha]^{25}_D$ −1.7° (*c* 0.47, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 210 (3.94), 240 (4.02), 304 (2.89) nm. IR (KBr): 3178, 2948, 2867, 2234, 1726, 1694, 1611 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.25 (1H, s), 3.60 (1H, d, J = 19.2 Hz), 2.91 (1H, d, J = 19.2 Hz), 2.68 (1H, s), 1.47, 1.30, 1.22, 1.15, 0.97, 0.94, 0.77 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 208.2, 197.7, 182.0, 171.8, 133.0, 130.5, 115.1, 113.9, 56.9, 52.0, 48.1, 45.2, 44.2, 44.0, 43.5, 41.1, 40.0, 36.7, 35.7, 33.1, 32.8, 32.4, 32.2, 27.6, 26.5, 24.2, 21.8, 20.1, 19.6, 19.3, 18.7. EIMS (70 eV) *m/z*: 491 $[M]^+$ (5.3), 461 (55), 445 (100), 351 (38), 310 (29), 257 (50). HREIMS Calcd for $C_{32}H_{41}O_4N$: 491.3036. Found: 491.3040. Anal. (Table 1).

Methyl 2-Cyano-3,11-dioxoursa-1,12-dien-28-oate (33). **33** was prepared from **70** according to the same method as for **25** to give a crystalline solid (90%): mp >275 °C dec; $[\alpha]^{25}_D$ +91° (*c* 0.36, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 250 (4.22) nm. IR (KBr): 2984, 2937, 2866, 2232, 1725, 1687, 1658, 1614 cm^{-1}. ^1H NMR (500 MHz, by a Varian Unityplus, $CDCl_3$): δ 8.55 (1H, s), 5.74 (1H, s), 3.63 (3H, s), 2.68 (1H, s), 2.49 (1H, d, J = 11.5 Hz), 2.12 (1H, m), 1.44, 1.34, 1.21, 1.15 (each 3H, s), 0.99 (3H, d, J = 6.4 Hz), 0.97 (3H, s), 0.89 (3H, d, J = 6.4 Hz). ^{13}C NMR (125.705 MHz, by a Varian Unityplus, $CDCl_3$): δ 197.9, 197.8, 177.2, 172.9, 165.6, 130.1, 115.1, 113.5, 54.2, 53.1, 52.1, 52.0, 47.8, 45.2, 45.1, 44.4, 39.9, 38.8, 36.0, 32.1, 30.4, 28.6, 27.8, 24.0, 21.7, 21.2, 21.1, 19.6, 19.4, 18.2, 17.3. EIMS (70 eV) *m/z*: 505 $[M]^+$ (62), 490 (15), 446 (19), 445 (19), 430 (23), 411 (47), 256 (37), 217 (37), 189 (69), 119 (100). HREIMS Calcd for $C_{32}H_{43}O_4N$: 505.3192. Found: 505.3200. Anal. (Table 1).

2-Cyano-3,11-dioxoursa-1,12-dien-28-oic Acid (34).[35] A mixture of **33** (155 mg, 0.31 mmol) and LiI (750 mg) in dry DMF (2.4 mL) was heated under reflux for 1.5 h. The reaction mixture was poured into water to give a solid. The solid was filtered and washed with water (several times). The crude solid (140 mg) was crystallized from a mixture of hexanes and EtOAc (2:1) to give **34** as crystals (90 mg, 60%). An analytically pure sample was obtained by crystallization from a mixture of CH_2Cl_2 and MeOH as crystals: mp >285 °C dec; $[\alpha]^{25}_D$ +119° (*c* 0.25, DMSO). UV (DMSO) λ_{max} (log ϵ): 264 (4.16) nm. IR (KBr): 3117, 3050, 2983, 2951, 2930, 2873, 2231, 1719, 1685, 1624 cm^{-1}. ^1H NMR [DMSO-d_6, internal standard: δ 2.50 (CD_2HSOCD_3)]: δ 8.49 (1H, s), 5.54 (1H, s), 2.95 (1H, s), 2.33 (1H, d, J = 11.2 Hz), 2.11 (1H, dd, J = 3.9, 13.2, 13.2 Hz), 1.35, 1.30, 1.13, 1.06 (each 3H, s), 0.942 (3H, d, J = 4.2 Hz), 0.935 (3H, s), 0.84 (3H, d, J = 6.4 Hz). EIMS (70 eV) *m/z*: 465 $[M - CN]^+$ (36), 446 $[M - CO_2H]^+$ (100), 420 (4.0), 405 (11), 315 (17), 244 (19). HREIMS Calcd for $C_{31}H_{41}O_4N - CN$: 465.3005. Found: 465.3010. Calcd for $C_{31}H_{41}O_4N - CO_2H$: 446.3059. Found: 446.3060. Anal. (Table 1).

Methyl 2-Aminocarbonyl-3,12-dioxooleana-1,9(11)-dien-28-oate (36). A solution of **27** (41.5 mg, 0.78 mmol) in saturated ammonia MeOH (4 mL) was kept at room temperature overnight. The mixture was evaporated in vacuo to give a residue (41 mg). The residue was subjected to flash column chromatography [hexanes−EtOAc (1:1.5)] to give **27** (18.5 mg) and **36** as an amorphous solid (19.6 mg; 49%, 88% based on recovered **27**): $[\alpha]^{24}_D$ +42° (*c* 0.36, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 242 (4.23) nm. IR (KBr): 3433, 3334, 2949, 2871, 1725, 1692, 1666 cm^{-1}. ^1H NMR ($CDCl_3$): δ 8.64 (1H, s), 8.35 (1H, d, J = 3.3 Hz), 6.22 (1H, s), 5.73 (1H, d, J = 3.3 Hz), 3.69 (3H, s), 3.05 (1H, ddd, J = 3.7, 4.5, 13.2 Hz), 2.92 (1H, d, J = 4.5 Hz), 1.41, 1.32 (each 3H, s), 1.20, 1.01 (each 6H, s), 0.90 (3H, s). ^{13}C NMR ($CDCl_3$): δ 204.4, 199.2, 178.5, 169.9, 165.3, 164.8, 127.8, 125.1, 52.1, 50.0, 47.8, 47.4, 46.2, 45.8, 42.2, 42.0, 35.9, 34.7, 33.4, 33.0, 31.73, 31.70, 30.8, 28.4, 28.2, 27.7, 24.7, 23.3, 22.9, 21.9, 21.8, 18.8. EIMS (70 eV) *m/z*: 523 $[M]^+$ (2.2), 508 (9.1), 506 (21), 446 (9.6), 315 (6.9), 84 (100). HREIMS Calcd for $C_{32}H_{45}O_5N$: 523.3298. Found: 523.3292. Anal. (Table 1).

Methyl 2-Formyl-3,12-dioxooleana-1,9(11)-dien-28-oate (37). **37** was prepared from **62** according to the same method as for **27** to give an amorphous solid (62%, 74% based on recovered **62**): $[\alpha]^{24}_D$ −3.7° (*c* 0.39, $CHCl_3$). UV (EtOH) λ_{max} (log ϵ): 254 (4.05) nm. IR (KBr): 2944, 2867, 1722, 1704, 1668, 1611 cm^{-1}. ^1H NMR ($CDCl_3$): δ 10.02 (1H, s), 8.11 (1H, s), 6.14 (1H, s), 3.70 (3H, s), 3.05 (1H, ddd, J = 3.7, 4.5, 13.2 Hz), 2.93 (1H, d, J = 4.5 Hz), 1.44, 1.33, 1.23, 1.19 (each 3H, s), 1.00 (6H, s), 0.89 (3H, s). ^{13}C NMR ($CDCl_3$): δ 202.2, 199.3, 189.8, 178.4, 169.9, 161.4, 131.5, 124.7, 52.1, 49.9, 48.2, 47.4, 46.0, 45.4, 42.3, 42.1, 36.0, 34.7, 33.4, 33.0, 31.9, 31.7, 30.8, 28.2, 27.5, 27.2, 24.7, 23.3, 22.8, 21.8, 21.6, 18.7. EIMS (70 eV) *m/z*: 508 $[M]^+$ (37), 493 (35), 446 (44), 315 (28), 84 (100). HREIMS Calcd for $C_{32}H_{44}O_5$: 508.3189. Found: 508.3183. Anal. (Table 1).

Methyl 3β-Hydroxy-11-oxours-12-en-28-oate (47). A solution of methyl 3β-acetoxy-11-oxours-12-en-28-oate (**46**)[10] (150 mg, 0.29 mmol) and KOH (1.0 g) in MeOH (10 mL) was heated under reflux for 30 min. After removal of MeOH in vacuo, the resultant mixture was acidified with 6 M aqueous HCl solution. The aqueous layer was extracted with a mixture of CH_2Cl_2 and Et_2O (1:2) (three times). The extract was worked up according to the standard method to give **47** as an amorphous solid (138 mg, quantitative): UV (EtOH) λ_{max} (log ϵ): 250 (4.17) nm. IR (KBr): 3494, 2928, 2869, 1728, 1660 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.59 (1H, s), 3.60 (3H, s), 3.21 (1H, dd, J = 5.9, 10.6 Hz), 2.78 (1H, ddd, J = 3.5, 3.5, 13.6 Hz), 2.41 (1H, d, J = 11.4 Hz), 2.29 (1H, s), 2.07 (1H, m), 1.29, 1.11, 0.99 (each 3H, s), 0.96 (3H, d, J = 6.2 Hz), 0.90 (3H, s), 0.86 (3H, d, J = 6.2 Hz), 0.79 (3H, s). ^{13}C NMR ($CDCl_3$): δ 200.1, 177.4, 163.0, 130.9, 78.9, 61.7, 55.2, 52.9, 52.0, 47.9, 44.8, 43.9, 39.34, 39.28, 38.82, 38.77, 37.3, 36.2, 33.2, 30.5, 28.6, 28.3, 27.5, 24.1, 21.3, 21.2, 19.0, 17.6, 17.3, 16.4, 15.8. EIMS (70 eV) *m/z*: 484 $[M]^+$ (40), 317 (100), 276 (48), 257 (34). HREIMS Calcd for $C_{31}H_{48}O_4$: 484.3553. Found: 484.3552. This material was used for the next reaction without further purification.

Methyl 3,11-Dioxours-12-en-28-oate (48). To a solution of **47** (144 mg, 0.30 mmol) in acetone (14 mL) in an ice bath was added Jones reagent dropwise until the color of the solution changed to pale brown from green. The mixture was stirred at room temperature for 10 min. After removal of acetone, water was added to the resultant mixture. The aqueous mixture was extracted with a mixture of CH_2Cl_2 and Et_2O (1:2) (three times). The extract was worked up according to the standard method to give **48** as an amorphous solid (128 mg, 89%): UV (EtOH) λ_{max} (log ϵ): 252 (4.11) nm. IR (KBr): 2949, 2869, 1726, 1709, 1654 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.65 (1H, s), 3.63 (3H, s), 2.96 (1H, ddd, J = 4.2, 7.1, 13.4 Hz), 2.65 (1H, ddd, J = 7.1, 11.2, 15.9 Hz), 2.45 (1H, d, J = 11.5 Hz), 2.40 (1H, s), 2.37 (1H, ddd, J = 4.2, 6.5, 15.9 Hz), 2.10 (1H, ddd, J = 4.6, 14.7, 14.7 Hz), 1.31, 1.26, 1.10, 1.06 (each 3H, s), 0.98 (3H, d, J = 6.3 Hz), 0.95 (3H, s), 0.88 (3H, d, J = 6.3 Hz). ^{13}C NMR ($CDCl_3$): δ 217.5, 199.3, 177.4, 163.6, 130.7, 60.9, 55.6, 52.9, 52.1, 47.9, 47.8, 44.7, 44.0, 39.9, 38.8, 36.9, 36.1, 34.4, 32.6, 30.5, 28.6, 26.6, 24.1, 21.6, 21.2, 21.1, 18.9, 17.3, 15.7. EIMS (70 eV) *m/z*: 482 $[M]^+$ (25), 467 (20), 423 (10), 317 (100), 276 (47), 257 (74). HREIMS Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3400. This material was used for the next reaction without further purification.

Methyl 3β-Hydroxy-12-oxooleanan-28-oate (50). 50 was prepared from methyl 3β-acetoxy-12-oxooleanan-28-oate (**49**)[12] according to the same method as for **47** to give a crystalline solid (quantitative): mp 133–135 °C. IR (KBr): 3540, 2945, 2866, 1725, 1698 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.67 (3H, s), 3.18 (1H, dd, J = 5.0, 10.9 Hz), 2.77 (1H, ddd, J = 3.4, 4.2, 13.4 Hz), 2.60 (1H, d, J = 4.2 Hz), 2.14 (2H, m), 1.84 (2H, m), 0.98, 0.96, 0.95, 0.93, 0.89, 0.84, 0.77 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 212.0, 178.6, 78.8, 55.3, 52.0, 49.9, 47.5, 42.1, 41.4, 39.0, 38.7, 38.1, 37.1, 36.4, 34.6, 33.6, 33.1, 32.1, 32.0, 30.8, 28.1, 27.7, 27.2, 23.3, 22.9, 20.7, 18.5, 16.3, 15.5, 15.4. EIMS (70 eV) *m/z*: 486 $[M]^+$ (37), 471 (100), 411 (65), 278 (68), 218 (65). HREIMS Calcd for $C_{31}H_{50}O_4$: 486.3709. Found: 486.3701.

Methyl 3,12-Dioxooleanan-28-oate (51). 51 was prepared from **50** according to the same method as for **48** to give an amorphous solid (98%): IR (KBr): 2948, 2866, 1723, 1702 cm^{-1}. ^1H NMR ($CDCl_3$): δ 3.69 (3H, s), 2.80 (1H, ddd, J = 3.7, 4.4, 13.7 Hz), 2.64 (1H, d, J = 4.4 Hz), 2.53 (1H, ddd, J = 7.2, 10.9, 15.9 Hz), 2.40 (1H, ddd, J = 3.8, 7.0, 15.9 Hz), 2.23 (2H, m), 1.09, 1.05, 1.01, 0.983, 0.976, 0.95, 0.90 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 217.1, 211.4, 178.6, 55.1, 52.0, 49.4, 47.6, 47.5, 42.2, 41.4, 38.8, 36.8, 36.4, 34.6, 34.1, 33.6, 33.1, 32.2, 31.3, 30.8, 27.8, 26.4, 23.3, 22.9, 21.4, 20.7, 19.7, 16.1, 15.0. EIMS (70 eV) *m/z*: 484 $[M]^+$ (4.2), 469 (39), 409 (100), 357 (6.7), 278 (25), 218 (72). HREIMS Calcd for $C_{31}H_{48}O_4$: 484.3553. Found: 484.3544.

Methyl 3β-Hydroxy-12-oxoolean-9(11)-en-28-oate (53). 53 was prepared from methyl 3β-acetoxy-12-oxoolean-9(11)-en-28-oate (**52**)[14] according to the same method as for **47** to give an amorphous solid (97%): UV (EtOH) λ_{max} (log ϵ): 250 (4.03) nm. IR (KBr): 3549, 3382, 2941, 2865, 1717, 1706, 1654, 1644, 1595 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.75 (1H, s), 3.68 (3H, s), 3.21 (1H, dd, J = 4.8, 11.4 Hz), 3.02 (1H, ddd, J = 3.5, 4.6, 13.4 Hz), 2.84 (1H, d, J = 4.6 Hz), 1.23, 1.18, 1.03 (each 3H, s), 0.99 (6H, s), 0.89, 0.83 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 200.8, 178.6, 178.5, 122.9, 78.2, 52.0, 50.4, 49.6, 47.5, 45.5, 41.9, 40.2, 39.4, 36.6, 36.0, 34.7, 33.5, 33.1, 33.0, 31.7, 30.8, 28.3, 27.7, 24.0, 23.9, 23.3, 22.9, 22.0, 18.2, 15.8. EIMS (70 eV) *m/z*: 484 $[M]^+$ (4.7), 469 (33), 409 (61), 407 (85), 315 (16), 278 (36), 218 (100). HREIMS Calcd for $C_{31}H_{48}O_4$: 484.3553. Found: 484.3553.

Methyl 3,12-Dioxoolean-9(11)-en-28-oate (54). 54 was prepared from **53** according to the same method as for **48** to give an amorphous solid (92%). An analytically pure sample was obtained by flash column chromatography [hexanes–EtOAc (3:1)]: UV (EtOH) λ_{max} (log ϵ): 250 (3.74) nm. IR (KBr): 2944, 2867, 1722, 1708, 1661, 1594 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.80 (1H, s), 3.70 (3H, s), 3.04 (1H, ddd, J = 3.3, 4.9, 13.2 Hz), 2.89 (1H, d, J = 4.9 Hz), 2.66 (1H, ddd, J = 7.2, 10.9, 15.7 Hz), 2.49 (1H, ddd, J = 3.8, 7.1, 15.7 Hz), 2.22 (1H, ddd, J = 3.9, 7.1, 13.4 Hz), 1.31, 1.28, 1.13, 1.09, 1.010, 1.005, 0.90 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 216.1, 200.3, 178.5, 176.8, 124.2, 52.0, 51.1, 49.7, 47.7, 47.5, 45.6, 42.0, 39.6, 37.2, 36.0, 34.7, 34.3, 33.5, 33.0, 32.2, 31.7, 30.8, 28.3, 26.4, 24.0, 23.8, 23.3, 22.9, 21.8, 21.6, 19.3. EIMS (70 eV) *m/z*: 482 $[M]^+$ (16), 467 (56), 423 (13), 407 (23), 315 (100), 255 (62), 246 (63). HREIMS Calcd for $C_{31}H_{46}O_4$: 482.3396. Found: 482.3392.

3β-Hydroxyolean-9(11)-en-28-oic Acid (57).[35] A mixture of **52** (2.27 g, 4.31 mmol), KOH (22 g), and anhydrous hydrazine (98%) (25 mL) in diethylene glycol (200 mL) was heated under reflux (inside temperature, 165 °C) for 1.5 h. Excess hydrazine was distilled off from the mixture until the inside temperature rose to 215 °C. Then, the mixture was heated under reflux (inside temperature, 215–220 °C) for 6 h. The mixture was poured into water (500 mL). Aqueous HCl solution (6 M) was added to give a precipitate. The precipitate (dry weight, 1.76 g) was filtered and washed with water (several times). The filtrate was extracted with a mixture of CH_2Cl_2 and Et_2O (1:2) (three times). The extract was worked up according to the standard method to give a solid (0.36 g). The combined solids were crystallized from a mixture of CH_2Cl_2 and MeOH (1:1) to afford **57** as colorless crystals (first crop, 670 mg; second crop, 180 mg). The solid obtained from the mother liquid was subjected to flash column chromatography [hexanes–EtOAc (2:1)] to give **57** as crystalline solid (200 mg, total weight: 1050 mg; 53%): mp >275 °C dec. IR (KBr): 3467, 3305, 2947, 2875, 1692 cm^{-1}. ^1H NMR [acetone-d_6, internal standard: δ 2.05 (CD_2HCOCD_3)]: δ 5.35 (1H, t, J = 3.7 Hz), 3.11 (1H, dd, J = 6.8, 9.0 Hz), 1.14, 1.11, 0.98, 0.94, 0.93, 0.89, 0.78 (each 3H, s). EIMS (70 eV) *m/z*: 456 $[M]^+$ (32), 441 (15), 302 (16), 248 (100). HREIMS Calcd for $C_{30}H_{48}O_3$: 456.3603. Found: 456.3603.

3-Oxoolean-9(11)-en-28-oic Acid (58). 58 was prepared from **57** according to the same method as for **48** to give an amorphous solid (95%): IR (KBr): 2947, 2870, 1708, 1694 cm^{-1}. ^1H NMR ($CDCl_3$): δ 5.38 (1H, t, J = 3.4 Hz), 2.63 (1H, ddd, J = 7.1, 11.5, 15.9 Hz), 2.42 (1H, ddd, J = 3.7, 6.8, 15.9 Hz), 1.25, 1.13, 1.09, 0.97, 0.94 (each 3H, s), 0.90 (6H, s). ^{13}C NMR ($CDCl_3$): δ 217.9, 185.2, 152.5, 118.7, 52.8, 48.1, 47.7, 43.8, 38.7, 38.6, 38.4, 36.2, 35.7, 34.9, 34.4, 33.7, 33.6, 33.2, 31.8, 30.8, 28.6, 27.3, 26.3, 25.5, 24.9, 23.6, 23.5, 21.5, 19.6, 18.7. EIMS (70 eV) *m/z*: 454 $[M]^+$ (32), 439 (13), 408 (26), 248 (65), 235 (100). HREIMS Calcd for $C_{30}H_{46}O_3$: 454.3447. Found: 454.3439.

Methyl 3-Oxooleana-1,11,13(18)-trien-28-oate (60). 60 was prepared from methyl 3-oxooleana-11,13(18)-dien-28-oate (**59**)[17] according to the same method as for **9**. The crude solid was subjected to flash column chromatography [hexanes–EtOAc (6:1)] to give **60** as a crystalline solid (66%): mp 131–133 °C. UV (EtOH) λ_{max} (log ϵ): 246 (4.54), 252 (4.54) nm. IR (KBr): 3029, 2944, 2859, 1726, 1674 cm^{-1}. ^1H NMR ($CDCl_3$): δ 7.27 (1H, d, J = 10.3 Hz), 6.57 (1H, dd, J = 2.9, 10.5 Hz), 5.89 (1H, d, J = 10.3 Hz), 5.79 (1H, dd, J = 1.7, 10.5 Hz), 3.68 (3H, s), 2.54 (1H, d, J = 14.4 Hz), 2.28 (2H, m), 1.91 (1H, m),

1.18, 1.17, 1.10, 0.98, 0.95, 0.86, 0.81 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 205.5, 177.1, 159.1, 136.2, 133.2, 126.7, 125.7, 125.0, 53.4, 52.0, 48.6, 48.4, 45.0, 42.4, 41.7, 40.8, 39.3, 37.0, 35.6, 32.8, 32.4, 32.0, 27.7, 25.2, 24.3, 21.32, 21.27, 20.0, 19.2, 16.7. EIMS (70 eV) *m/z*: 464 $[M]^+$ (84), 449 (13), 405 (100), 327 (14), 267 (19), 239 (29). HREIMS Calcd for $C_{31}H_{44}O_3$: 464.3290. Found: 464.3293.

Methyl 2-Hydroxymethylene-3,12-dioxoolean-9(11)-en-28-oate (62). To a solution of **54** (4.00 g, 8.29 mmol) in dry benzene (90 mL) was added ethyl formate (97%) (3.0 mL) and NaOMe (2.68 g, 50 mmol). The mixture was stirred at room temperature for 2 h. Then the mixture was diluted with a mixture of CH_2Cl_2 and Et_2O (1:2) and washed with 5% aqueous HCl solution (three times). The washings were reextracted with a mixture of CH_2Cl_2 and Et_2O (1:2) and the combined organic layers were worked up according to the standard method to give **62** as an amorphous solid (4.19 g, 99%): UV (EtOH) λ_{max} (log ϵ): 252 (3.66), 294 (3.53) nm. IR (KBr): 3461, 2950, 2867, 1724, 1661, 1596 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 14.86 (1H, d, J = 2.8 Hz), 8.77 (1H, d, J = 2.8 Hz), 5.90 (1H, s), 3.70 (3H, s), 3.05 (1H, ddd, J = 3.1, 4.5, 13.6 Hz), 2.92 (1H, d, J = 4.5 Hz), 2.62 (1H, d, J = 14.4 Hz), 2.30 (1H, d, J = 14.4 Hz), 1.28, 1.24, 1.18, 1.17, 1.02, 1.01, 0.91 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 200.3, 190.2, 188.3, 178.5, 175.8, 124.4, 105.1, 52.1, 49.7, 48.4, 47.5, 45.6, 42.0, 40.6, 39.3, 37.2, 36.0, 34.7, 33.5, 33.0, 31.7, 31.5, 30.8, 28.5, 28.4, 23.6, 23.3, 23.2, 22.9, 21.8, 21.0, 19.1. EIMS (70 eV) *m/z*: 510 $[M]^+$ (11), 495 (39), 435 (38), 315 (100), 255 (55). HREIMS Calcd for $C_{32}H_{46}O_5$: 510.3345. Found: 510.3351. This material was used for the next reaction without further purification.

Methyl 12-Oxoisoxazolo[4,5-*b*]olean-9(11)-en-28-oate (63). To a solution of **62** (4.00 g, 7.83 mmol) in EtOH (110 mL) and water (11 mL) was added hydroxylamine hydrochloride (5.44 g, 78 mmol). The mixture was heated under reflux for 1 h. The mixture was concentrated in vacuo and water (50 mL) was added. The mixture was extracted with EtOAc (three times). The combined organic layers were washed with water (three times) and saturated aqueous NaCl solution (three times), dried over $MgSO_4$, and filtered. The filtrate was evaporated in vacuo to give a solid. The solid was subjected to flash column chromatography [hexanes−EtOAc (3:1)] to give **63** as an amorphous solid (2.63 g, 66%): UV (EtOH) λ_{max} (log ϵ): 238 (3.63) nm. IR (KBr): 2944, 2867, 1724, 1660, 1596 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 8.07 (1H, s), 5.89 (1H, s), 3.70 (3H, s), 3.05 (1H, ddd, J = 3.7, 4.6, 13.4 Hz), 2.93 (1H, d, J = 4.6 Hz), 2.79 (1H, d, J = 15.1 Hz), 2.40 (1H, d, J = 15.1 Hz), 1.35, 1.29, 1.27, 1.16, 1.03, 1.01, 0.90 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 200.2, 178.5, 176.3, 172.3, 150.4, 124.7, 108.7, 52.1, 49.9, 49.7, 47.5, 45.8, 42.0, 41.5, 36.1, 35.4, 34.7, 33.8, 33.5, 33.0, 31.7, 31.5, 30.9, 29.0, 28.4, 24.8, 23.29, 23.25, 22.9, 21.8, 21.6, 18.5. EIMS (70 eV) *m/z*: 507 $[M]^+$ (14), 492 (51), 446 (25), 432 (49), 315 (100). HREIMS Calcd for $C_{32}H_{45}O_4N$: 507.3349. Found: 507.3354.

Methyl 2-Cyano-3,12-dioxoolean-9(11)-en-28-oate (64). To a solution of **63** (2.00 g, 3.94 mmol) in MeOH (60 mL) and Et_2O (125 mL) in an ice bath was added NaOMe (7.25 g, 134 mmol). The mixture was stirred at room temperature for 45 min and then diluted with a mixture of CH_2Cl_2 and Et_2O (1:2). It was washed with 5% aqueous HCl solution (three times) and the acidic washings were reextracted with a mixture of CH_2Cl_2 and Et_2O (1:2). The combined organic layers were worked up according to the standard method to give **64** as an amorphous solid (2.00 g, quantitative): UV (EtOH) λ_{max} (log ϵ): 242 (4.16) nm. IR (KBr): 3411, 2944, 2867, 2206, 1722, 1661, 1636, 1597 cm^{-1}. ^{1}H NMR of major tautomer **64a** ($CDCl_3$): δ 7.08 (1H, brs), 5.75 (1H, s), 3.67 (3H, s), 3.01 (1H, ddd, J = 3.7, 4.6, 13.7 Hz), 2.89 (1H, d, J = 4.6 Hz), 2.40 (1H, d, J = 15.3 Hz), 2.23 (1H, d, J = 15.3 Hz), 1.24, 1.21, 1.19, 1.11 (each 3H, s), 0.98 (6H, s), 0.88 (3H, s). EIMS (70 eV) *m/z*: 507 $[M]^+$ (84), 492 (99), 432 (58), 315 (100). HREIMS Calcd for $C_{32}H_{45}O_4N$: 507.3349. Found: 507.3340. This material was used for the next reaction without further purification.

Methyl 2-Hydroxymethylene-3,11-dioxoolean-12-en-28-oate (65). 65 was prepared from **45** according to the same method as for **62** to give a crystalline solid (98%): mp 232−234 °C. UV (EtOH) λ_{max} (log ϵ): 254 (4.15), 296 (3.91) nm. IR (KBr): 3456, 2944, 2867, 1728, 1656, 1589 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 14.87 (1H, d, J = 2.7 Hz), 8.62 (1H, d, J = 2.7 Hz), 5.69 (1H, s), 3.64 (3H, s), 3.49 (1H, d, J = 14.8 Hz), 3.03 (1H, dd, J = 3.6, 13.9 Hz), 2.40 (1H, s), 2.05 (1H, ddd, J = 4.1, 13.7, 13.7 Hz), 1.93 (1H, d, J = 14.8 Hz), 1.36, 1.18, 1.12, 1.08, 0.95, 0.94, 0.93 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 199.9, 189.6, 189.2, 177.6, 169.6, 128.0, 106.0, 59.8, 52.4, 52.1, 46.4, 44.8, 44.5, 43.8, 41.8, 40.2, 39.9, 36.5, 33.8, 33.0, 32.0, 31.7, 30.9, 28.6, 28.0, 23.64, 23.59, 23.1, 21.1, 18.8, 18.7, 14.8. EIMS (70 eV) *m/z*: 510 $[M]^+$ (14), 495 (21), 451 (22), 446 (42), 435 (22), 317 (31), 257 (100). HREIMS Calcd for $C_{32}H_{46}O_5$: 510.3345. Found: 510.3348.

Methyl 11-Oxoisoxazolo[4,5-*b*]olean-12-en-28-oate (66). 66 was prepared from **65** according to the same method as for **63** to give an amorphous solid (74%): UV (EtOH) λ_{max} (log ϵ): 250 (4.10) nm. IR (KBr): 2944, 2867, 1728, 1657, 1624 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 7.99 (1H, s), 5.71 (1H, s), 3.67 (1H, d, J = 15.5 Hz), 3.64 (3H, s), 3.04 (1H, dd, J = 3.8, 13.6 Hz), 2.51 (1H, s), 2.06 (1H, ddd, J = 4.2, 13.9, 13.9 Hz), 2.03 (1H, d, J = 15.5 Hz), 1.37, 1.31, 1.22, 1.06, 0.96, 0.94, 0.93 (each 3H, s). ^{13}C NMR ($CDCl_3$): δ 199.8, 177.6, 172.4, 169.6, 150.5, 128.1, 109.2, 60.3, 53.5, 52.1, 46.4, 45.1, 44.5, 43.8, 41.8, 38.7, 36.2, 34.9, 33.9, 33.1, 32.1, 31.7, 30.9, 29.1, 28.1, 23.7, 23.6, 23.1, 21.7, 18.7, 18.2, 15.8. EIMS (70 eV) *m/z*: 507 $[M]^+$ (31), 492 (30), 448 (20), 432 (28), 257 (72), 217 (100). HREIMS Calcd for $C_{32}H_{45}O_4N$: 507.3349. Found: 507.3345.

Methyl 2-Cyano-3,11-dioxoolean-12-en-28-oate (67). 67 was prepared from **66** by the similar method as for **64**. The crude solid was subjected to flash column chromatography [hexanes−EtOAc (2:1)] to give **67** as an amorphous solid (92%): UV (EtOH) λ_{max} (log ϵ): 246 (4.18) nm. IR (KBr): 3411, 2944, 2867, 2200, 1725, 1656 cm^{-1}. ^{1}H NMR of major tautomer **67a** ($CDCl_3$): δ 6.40 (1H, brs), 5.67 (1H, s), 3.62 (3H, s), 3.33 (1H, d, J = 15.9 Hz), 3.02 (1H, dd, J = 3.7, 13.7 Hz), 2.53 (1H, s), 2.36 (1H, d, J = 15.9 Hz), 1.33, 1.15, 1.11, 1.08 (each 3H, s), 0.92 (6H, s), 0.87 (3H, s). EIMS (70 eV) *m/z*: 507 $[M]^+$ (3.7), 492 (5.2), 447 (5.8), 432 (8.4), 276 (7.0), 257 (21), 217 (31), 84 (100). HREIMS Calcd for $C_{32}H_{45}O_4N$: 507.3349. Found: 507.3349.

Methyl 2-Hydroxymethylene-3,11-dioxours-12-en-28-oate (68). 68 was prepared from **48** according to the same method as for **62** to give an amorphous solid (89%): UV (EtOH) λ_{max} (log ϵ): 254 (4.06), 298 (3.84) nm. IR (KBr): 3454, 2978, 2931, 2866, 1728, 1659, 1619, 1590 cm^{-1}. ^{1}H NMR (500 MHz, by a Varian Unityplus, $CDCl_3$): δ 14.87 (1H, d, J = 3.2 Hz), 8.63 (1H, d, J = 3.2 Hz), 5.67 (1H, s), 3.63 (3H, s), 3.46 (1H, d, J = 14.9 Hz), 2.46 (1H, d, J = 11.2 Hz), 2.40 (1H, s), 2.10 (1H, m), 1.98 (1H, d, J = 14.9 Hz), 1.31, 1.20, 1.13, 1.12 (each 3H, s), 0.98 (1H, d, J = 6.3 Hz), 0.96 (3H, s), 0.88 (3H, d, J = 6.6 Hz). ^{13}C NMR (125.705 MHz, by a Varian Unityplus, $CDCl_3$): δ 199.4, 189.7, 189.2, 177.4, 163.7, 130.9, 106.0, 59.5, 53.0, 52.4, 52.1, 47.9, 44.4, 44.0, 40.2, 40.0, 38.9, 38.8, 36.5, 36.1, 32.2, 30.5, 28.7, 28.6, 24.1, 21.2, 21.1, 18.9, 18.7, 17.3, 14.9. EIMS (70 eV) *m/z*: 510 $[M]^+$ (15), 495 (48), 435 (42), 315 (100), 274 (22), 255 (57). HREIMS Calcd for $C_{32}H_{46}O_5$: 510.3345. Found: 510.3347.

Methyl 11-Oxoisoxazolo[4,5-*b*]urs-12-en-28-oate (69). 69 was prepared from **68** according to the same method as for **63** to give an amorphous solid (81%): UV (EtOH) λ_{max} (log ϵ): 248 (4.09) nm. IR (KBr): 2973, 2937, 2866, 1727, 1658, 1619 cm^{-1}. ^{1}H NMR (500 MHz, by a Varian Unityplus, $CDCl_3$): δ 7.99 (1H, s), 5.68 (1H, s), 3.64 (1H, d, J = 15.6 Hz), 3.63 (3H, s), 2.50 (1H, s), 2.46 (1H, d, J = 11.5 Hz), 2.11 (1H, m), 2.07 (1H, d, J = 15.6 Hz), 1.33, 1.31, 1.23, 1.09 (each 3H, s), 0.98 (3H, d, J = 6.6 Hz), 0.97 (3H, s), 0.89 (3H, d, J = 6.6 Hz). ^{13}C NMR (125.705 MHz, by a Varian Unityplus, $CDCl_3$): δ 199.2, 177.3, 172.4, 163.7, 150.5, 130.8, 109.2, 60.0, 53.5, 52.9, 52.1, 47.8, 44.7, 44.0, 38.9, 38.8, 38.6, 36.2, 36.1, 34.9, 32.3, 30.5, 29.1, 28.7, 24.1, 21.7, 21.2, 21.1, 18.7, 18.2, 17.3, 15.8. EIMS (70 eV) *m/z*: 507 $[M]^+$ (9.3), 492 (13), 317 (13), 257 (24), 217 (12), 84 (100). HREIMS Calcd for $C_{32}H_{45}O_4N$: 507.3349. Found: 507.3351.

Methyl 2-Cyano-3,11-dioxours-12-en-28-oate (70). 70 was prepared from **69** by the similar method as for **64**. The crude solid was subjected to flash column chromatography [hexanes−EtOAc (2:1)] to give **70** as a crystalline solid (94%): mp 169−171 °C. UV (EtOH) λ_{max} (log ϵ): 246 (4.17) nm. IR (KBr): 3401, 2978, 2937, 2866, 2202, 1725, 1668 cm^{-1}. ^1H NMR of major tautomer **70a** (500 MHz, by a Varian Unityplus, $CDCl_3$): δ 5.86 (1H, brs), 5.66 (1H, s), 3.62 (3H, s), 3.33 (1H, d, J = 15.7 Hz), 2.45 (1H, d, J = 10.3 Hz), 2.33 (1H, s), 2.10 (1H, m), 1.92 (1H, d, J = 15.7 Hz), 1.29, 1.17, 1.15, 1.09 (each 3H, s), 0.97 (3H, d, J = 6.4 Hz), 0.93 (3H, s), 0.87 (3H, d, J = 6.6 Hz). EIMS (70 eV) *m/z*: 507 $[M]^+$ (25), 492 (31), 467 (45), 446 (54), 317 (34), 276 (26), 257 (85), 217 (100). HREIMS Calcd for $C_{32}H_{45}O_4N$: 507.3349. Found: 507.3351.

Methyl 3-Hydroxy-2-methoxycarbonyl-12-oxooleana-2,9(11)-dien-28-oate (71). A mixture of **54** (258 mg, 0.53 mmol) and 1.8 M DMF solution of methoxymagnesium methyl carbonate (Stiles' reagent) (2.5 mL, 4.5 mmol) was heated at 110 °C for 1 h while a slow stream of N_2 was bubbled through the mixture with a pipet. To the mixture were added 5% aqueous HCl solution and EtOAc. The aqueous layer was extracted with EtOAc (three times). The combined organic layers were washed with water (three times) and saturated aqueous NaCl solution (three times), dried over $MgSO_4$, and filtered. The filtrate was evaporated in vacuo to give a solid (305 mg). To a solution of the solid in THF (6 mL) was added excessive amount of ethereal diazomethane. The mixture was kept at room temperature for 10 min. The mixture was evaporated in vacuo to give a solid (310 mg). The solid was subjected to flash column chromatography [hexanes−EtOAc (4:1)] to give **71** as crystals (225 mg, 78%): mp 210−211 °C. UV (EtOH) λ_{max} (log ϵ): 252 (4.20) nm. IR (KBr): 2944, 2867, 1725, 1661, 1618 cm^{-1}. ^1H NMR ($CDCl_3$): δ 12.49 (1H, s), 5.94 (1H, s), 3.76, 3.69 (each 3H, s), 3.04 (1H, ddd, J = 3.1, 4.9, 13.2 Hz), 2.90 (1H, d, J = 4.9 Hz), 2.70 (1H, d, J = 15.3 Hz), 2.06 (1H, d, J = 15.3 Hz), 1.26, 1.20, 1.17, 1.14 (each 3H, s), 1.00 (6H, s), 0.89 (3H, s). ^{13}C NMR ($CDCl_3$): δ 200.5, 178.5, 176.9, 176.7, 173.9, 124.5, 94.1, 52.0, 51.8, 49.7, 48.6, 47.5, 45.6, 42.0, 39.3, 38.6, 36.3, 36.1, 34.7, 33.5, 33.1, 31.7, 31.5, 30.8, 28.6, 28.4, 24.3, 23.3, 23.2, 22.9, 21.8, 20.4, 19.1. EIMS (70 eV) *m/z*: 540 $[M]^+$ (3.9), 525 (5.7), 508 (23), 493 (54), 433 (35), 315 (100). HREIMS Calcd for $C_{33}H_{48}O_6$: 540.3451. Found: 540.3454.

Evaluation Methods. 1. Reagents. Recombinant mouse IFN-γ (LPS content, <10 pg/mL) was purchased from Genzyme (Cambridge, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Inhibitory test compounds were dissolved in DMSO before addition to cell cultures; final concentrations of DMSO were 0.1% or less. Controls with DMSO alone were run in all cases.

2. Cell Culture. To obtain primary macrophages, female CD-1 mice, 5−10 weeks of age (Charles River Breeding Laboratories, Wilmington, MA), were injected intraperitoneally with 2 mL of 4% thioglycollate broth (Difco Laboratories, Detroit, MI). Four days after injection, peritoneal macrophages were harvested and processed according to Nathan's procedure.[4b] Cells were seeded in 96-well plates at 2 × 10^5 cells/well and incubated for 48 h with 20 ng/mL IFN-γ in the presence or absence of inhibitory test compounds

3. Measurement of NO Production in Mouse Macrophages. Nitrite accumulation was used as an indicator of NO production in the medium and was assayed by the Griess reaction.[4a] Griess reagent (100 μL) was added to 100 μL of each supernatant from IFN-γ or inhibitory test compound-treated cells in triplicate. The protein determination was performed by Bradford protein assay. The plates were read at 550 nm against a standard curve of sodium nitrite.

Acknowledgment. We thank Drs. Carl Nathan and Qiao-wen Xie for expert advice on the preparation of macrophages and the nitric oxide assay. We also thank Dr. Steven Mullen (University of Illinois), Dr. Mary K. Young and Mr. Ron New (UC Riverside), Dr. Stephen W. Wright (Pfizer), Dr. Timothy C. Barden (American Cyanamid), and Dr. Mark G. Saulnier (Bristol-Myers Squibb) for the mass spectra and also Prof. David A. Evans and Mr. Brett D. Allison (Harvard University) for the optical rotation measurements. This investigation was supported by funds from NIH Grant 1 R01-CA78814, the Norris Cotton Cancer Center, U.S. Department of Defense Grants DAMD17-96-1-6163, DAMD17-98-1-8604, and DAMD17-99-1-9168, the Oliver and Jennie Donaldson Charitable Trust, the National Foundation for Cancer Research, and a Zenith Award from the Alzheimer's Association. M.B.S. is Oscar M. Cohn Professor, F.G.F., Jr. is an Oscar M. Cohn Scholar, and Y.W. is a Howard Hughes Medical Institute Predoctoral Fellow.

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JM0002230

Prospects for prevention and treatment of cancer with selective PPARγ modulators (SPARMs)

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Peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor and transcription factor that regulates the expression of many genes relevant to carcinogenesis, is now an important target for development of new drugs for the prevention and treatment of cancer. Deficient expression of PPARγ can be a significant risk factor for carcinogenesis, although in some cases overexpression enhances carcinogenesis. Ligands for PPARγ suppress breast carcinogenesis in experimental models and induce differentiation of human liposarcoma cells. By analogy to the selective estrogen receptor modulator (SERM) concept, it is suggested that selective PPARγ modulators (SPARMs), designed to have desired effects on specific genes and target tissues without undesirable effects on others, will be clinically important in the future for chemoprevention and chemotherapy of cancer.

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Specific molecular targets have become important for the development of new agents for chemoprevention[1] and chemotherapy[2] of cancer. Members of the nuclear receptor superfamily (steroid receptors and their relatives) are excellent examples of such targets. This family includes estrogen receptors (ERs), retinoic acid receptors (RARs), retinoid X receptors (RXRs, whose selective ligands are now called 'rexinoids'), and the vitamin D receptor (VDR, whose ligands have been called 'deltanoids'). Ligands for these receptors have been used extensively to prevent or treat experimental and clinical breast cancer[3–12]. The latest superfamily addition to this battery is peroxisome proliferator-activated receptor γ (PPARγ). There is a strong mechanistic basis for this targeting, as the above nuclear receptors are all transcription factors controlling expression of a large number of genes relevant to the process of carcinogenesis[13].

As was the case for its heterodimeric partner (RXR), PPARγ was originally discovered[14] as an 'orphan'[15] receptor (a protein belonging to the nuclear receptor superfamily, but without a known ligand). Subsequently, it was found that the widely used anti-diabetic thiazolidinedione (TZD) drugs were ligands for PPARγ (Ref. 16), although the chemical structure of the endogenous ligand for this receptor remains undetermined. Practical applications in the treatment of diabetes have moved PPARγ studies to the forefront of both molecular and clinical therapeutics[17], and it has now become important to determine the role of PPARγ in other major chronic human diseases, such as atherogenesis and carcinogenesis. The latter topic is the subject of this review. Because the binding pocket of PPARγ for its ligands is exceptionally large[18], it is not surprising that several diverse classes of synthetic molecules can serve as functional ligands. Some of the examples discussed in this review are shown in Fig. 1.

PPARγ and susceptibility to carcinogenesis

The actual disease process in cancer is carcinogenesis[19], a chronic progression ending in invasive and metastatic disease. Thus, it is useful first to look at the role of PPARγ in the suppression of early stages of carcinogenesis, rather than to start with the potential of PPARγ ligands to cure invasive malignancies. Carcinogenesis is a result of aberrant differentiation, rather than an intrinsic disorder of the cell cycle, which cannot account for the phenomenon of invasiveness and metastasis. The landmark observation that PPARγ is a crucial transcription factor for the control of differentiation[20] provides the background for the following discussion of PPARγ and carcinogenesis.

Although there is currently scant evidence for a direct role of mutation of PPARγ in the causation of most common human carcinomas, such as those of lung, breast, prostate, pancreas, and ovary, there are two important exceptions. First, in a study of 55 patients with sporadic colon cancers[21], four *PPARγ* mutations were found (one nonsense, one frameshift, and two missense).

Second, it has been shown that thyroid follicular carcinomas frequently have a chromosomal translocation involving PPARγ, leading to the formation of the PAX8–PPARγ1 fusion oncoprotein[22]. Despite these observations, extensive investigation has failed to show that mutation of *PPARγ* is a common phenomenon in human carcinogenesis.

However, even without mutation of *PPARγ*, the loss of its expression could be an important risk factor for the development of carcinoma, because the gene encoding PPARγ might fail to be expressed, either because of epigenetic silencing (e.g. histone deacetylation, DNA methylation), or as a result of as yet unidentified post-translational mechanisms. Indeed, recent animal studies have shown that reduced expression of the *PPARγ* gene enhances carcinogenesis; *PPARγ* heterozygous (+/–) mice are

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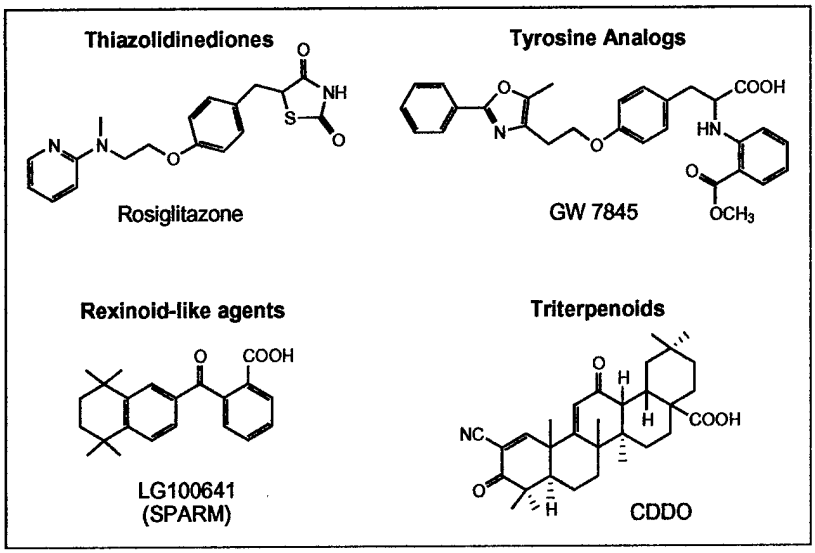


Fig. 1. Diversity of synthetic ligands for peroxisome proliferator-activated receptor γ (PPARγ). Abbreviations: CDDO, 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid; SPARM, selective PPAR modulator.

at a markedly enhanced risk for colon carcinogenesis after exposure to the classic colon carcinogen, azoxymethane[23]. By contrast, other recent animal studies have shown that constitutive overexpression of PPARγ increases the risk of breast cancer in mice already susceptible to the disease[24]. Although results of the above two studies appear to be paradoxical, we suggest that this conundrum might be resolved by careful dose-response studies, in which the level of *PPARγ* gene expression and signalling is carefully controlled.

Ultimately, it will be essential to determine the level of *PPARγ* gene expression and signalling in different human tissues at risk for carcinogenesis. Furthermore, it would not be surprising if individual PPARγ ligands gave different, tissue-specific results in different tissues in chemoprevention trials, just as tamoxifen suppresses mammary carcinogenesis but enhances uterine carcinogenesis in women[3,4,25]. Thus, the context of the genetic and epigenetic pathology in different premalignant tissues (and even in the same tissue under varying conditions) might be an important determinant of whether any particular PPARγ ligand suppresses or accelerates the process of carcinogenesis.

SERMs, SPARMs and chemoprevention of cancer

The simplistic concept that ligands for members of the nuclear receptor superfamily should be classified either as 'agonists' or as 'antagonists' is often misleading. For example, as noted above, it is erroneous to classify tamoxifen simply as an estrogen antagonist (an 'anti-estrogen'), as has often been done in the past. Although tamoxifen indeed acts *in vivo* as an estrogen antagonist in the breast, it has agonistic actions in bone and uterus[3,4,25]. Furthermore, even in the very same cell in culture, tamoxifen can exhibit opposite actions, depending on the cellular context; in MCF-7 breast cancer cells, tamoxifen is an estrogen agonist in the absence of estrogen, and an antagonist in its presence.

The context for all of these opposite actions is provided by numerous other components in the cellular machinery that interact with a particular ligand and its receptor, most notably the different co-activators and co-repressors that are crucial determinants of transcriptional activation and repression by the receptor[3,25–28]. Thus, the outmoded and simplistic concept of tamoxifen as an anti-estrogen has been replaced by the more useful selective estrogen receptor modulator (SERM) concept, which can account for the different contextual actions of tamoxifen, depending on the cellular milieu in which this drug acts[3,25,26].

This more realistic concept (i.e. that a ligand for a nuclear receptor can act as a modulator rather than as a simple agonist or antagonist), can now be extended to ligands for PPARγ and RXRs. Indeed, by analogy to the SERM concept, the term SPPARM (for selective PPAR-modulator) has been recently suggested as a useful acronym[29], although for convenience we will refer to such selective modulators as SPARMs. It is already known that new SERMs have many of the beneficial agonistic effects of estrogen on certain target tissues (bone, cardiovascular, brain), as well as being antagonistic to estrogen in cancer-susceptible organs such as breast and uterus[3,25,26]. In turn, the recent description of new selective modulators (contextual agonists or antagonists) of either PPARγ (Ref. 30) or RXRs (Ref. 31) is a major advance that presages new and unforeseen use of both PPARγ and RXR as targets for new drug discovery.

The contextual actions of ligands for nuclear receptors are hardly surprising, as it has been known for more than 15 years that the actions of many peptide growth factors and cytokines are strongly dependent on cellular context[32]. For example, transforming growth factor (TGF)-β can suppress early stages but accelerate later stages of carcinogenesis[33]. Elucidation of the unique tissue and organ specificity of new SPARMs and RXR modulators will undoubtedly involve detailed understanding of their interaction with their cognate receptors, and the unique manner in which ligand–receptor complexes interact with various co-activators and co-repressors. It is fortunate that, in the case of PPARγ, X-ray diffraction studies and computational protein modeling have already provided many of the required molecular details that will allow even further advances[18,34] (see Fig. 2).

The literature on the possible benefits and risks in using various PPARγ ligands to suppress carcinogenesis in experimental animals is still in its infancy, and not surprisingly there are conflicting results, mainly because investigators have used different agents at different doses in different test systems. The most clear-cut result reported thus far has been the suppression of mammary carcinogenesis by GW7845, a potent member of a

Fig. 2. Structure of peroxisome proliferator-activated receptor γ (PPARγ) (blue and red ribbon) bound to SRC-1 (yellow ribbon) and rosiglitazone (green carbons)[18]. Rosiglitazone (lower and center left) binds to a side-chain of the AF2 helix (top center, red ribbon), which in turn recruits the co-activator SRC-1 (upper right). Hydrogen, nitrogen, oxygen and sulfur atoms are shown in white, dark blue, red and yellow, respectively. Molecular model courtesy of Millard Lambert (GlaxoSmithKline).

new class of ligands for PPARγ (tyrosine analogs). In a classic animal model, rats were first treated with a single dose of the selective breast carcinogen nitrosomethylurea and then placed on diets containing either control vehicle or GW7845 for two months. This agent significantly reduced tumor incidence, tumor number, and tumor weight[35] (Fig. 3). By contrast, the PPARγ ligands, troglitazone and rosiglitazone, have been found to enhance colon polyp formation in *Min* mice, which have a mutation in the adenomatous polyposis coli (*APC*) tumor suppressor gene[36,37]. It should be noted that in these latter studies, an evaluation of dose/response to the PPARγ ligands was not performed.

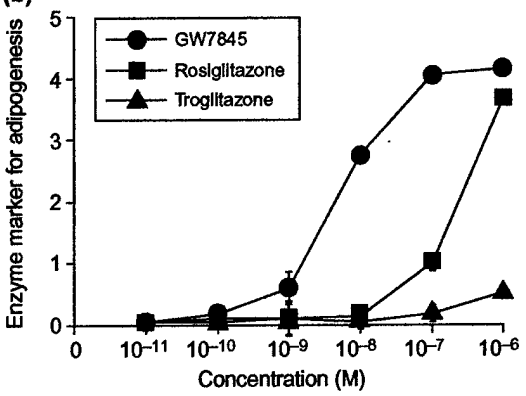
Although animal studies reveal much-needed insight, the data might not extrapolate to clinical practice, and in this regard, it is fortunate that a large clinical trial of the chemopreventive activity of PPARγ ligands is now underway, even though it was not planned as such. Just as the major clinical benefit of the SERM, raloxifene, for prevention of breast cancer was demonstrated in trials in which cancer prevention was not the primary endpoint, so too there is a very large group of men and women currently receiving long-term treatment with PPARγ ligands (thiazolidinediones, such as rosiglitazone and pioglitazone) for their type 2 diabetes.

Thus, if the appropriate clinical data relevant to carcinogenesis were to be collected for the long-term use of TZDs, and an appropriate control group were added, one could evaluate the hypothesis that a SPARM can lower the risk for the development of cancer. A similar strategy was used successfully to evaluate the effect of raloxifene for prevention of breast cancer in the multiple outcomes of raloxifene evaluation (MORE) trial[5,6]. Indeed, the landmark MORE trial has now led to the current study of tamoxifen and raloxifene (STAR) trial, in which the clinically useful SERM profiles of tamoxifen and raloxifene will be compared; tamoxifen is a risk

(a)

Compound	Murine PPARγ assay EC_{50} (μM)	Human PPARγ assay EC_{50} (μM)
Troglitazone (TZD)	0.78	0.55
Rosiglitazone (TZD)	0.076	0.043
GW7845	0.0012	0.00071

(b)



(c)

Treatment	Average tumour burden (g)	Rats with three or more tumours	Rats with tumour burden > 5g
Control	5.6	22/42	18/42
GW7845	1.6 ($P < 0.0001$)	2/42 ($P < 0.0001$)	3/42 ($P < 0.0003$)

Fig. 3. A potent new ligand for peroxisome proliferator-activated receptor γ (PPARγ) prevents breast cancer. (a) The tyrosine analog GW7845 is more potent than the TZDs troglitazone and rosiglitazone in molecular transactivation assays[17] (see Fig. 1 for structure). (b) GW7845 is more potent than TZDs in a cellular adipocyte-differentiation assay[35]. (c) GW7845 prevents breast cancer in rats[35].

factor for uterine carcinogenesis, whereas raloxifene is not[4,6]. Likewise, one might expect that different TZDs and other PPARγ ligands will also be found to have different SPARM profiles, based on their differential recruitment of co-activators and co-repressors.

The evaluation of TZDs, now widely used for the treatment of diabetes, as potential human cancer chemopreventive agents will be interesting, particularly for the wide variety of target organs in which PPARγ has been suggested to play a role in carcinogenesis, including in the breast, the prostate, and the colon. It has been reported that treatment of patients with the TZD troglitazone can lower serum prostate specific antigen (PSA) levels in prostate cancer patients[38,39]. Evaluation of the effects of long-term rosiglitazone and pioglitazone therapy (for type 2 diabetes) on the clinical progression of colon carcinogenesis will be particularly interesting, and could help to clarify some of the conflicting results from animal studies[23,36,37].

SPARMs and treatment of invasive cancer

The prospects for using SPARMs as therapeutic agents for treatment of common types of advanced human malignancies are still unclear. Although cell-culture studies, as well as experiments performed in mice, have shown that TZDs can suppress the growth of human tumor cell lines derived from the breast, prostate, and colon[39,46,49–51], clinical application remains to be determined. One rather spectacular result has been reported, namely the finding that human liposarcoma cells could be induced in culture by TZDs to differentiate into cells resembling mature adipocytes[40]. Analogous results have been obtained in patients with liposarcomas, which have a very poor clinical prognosis[41]. This is one of the first examples of the use of a pharmacological agent to induce the differentiation of malignant cells in a solid human tumor.

Another important area for differentiation therapy is the treatment of various myelogenous and monocytic leukemias, many of which also have poor prognosis. PPARγ is known to be expressed in cells of the myeloid–monocytic lineage[42,43]. Moreover, it has been shown that rosiglitazone, as well as the synthetic triterpenoid 2-cyano-3,12-dioxoolean-1,9-dien-28-oic acid (CDDO), which is also a PPARγ ligand, can induce differentiation in various human myeloid and monocytic leukemia cell lines[42,44,45].

ER-negative breast cancer, which has a notably poor prognosis, is yet another important target for the use of SPARMs. It has been shown that TZDs induce proteins characteristic of the differentiated state in human ER-negative breast-cancer cell lines[46]. However, some ER-negative cancer cells are refractory to this treatment, presumably because of high MAP kinase activity, which leads to phosphorylation of PPARγ. Phosphorylated PPARγ has greatly reduced transcriptional activity and ability to promote differentiation[47,48], but this block can be overcome by the use of a MAP kinase inhibitor[46]. Thus, a combination of two drugs with a direct mechanistic rationale for their combined use could provide clinical benefit.

Treatment of invasive cancer with SPARMs is a new area, and many of the studies performed thus far have used a low-potency agent such as troglitazone, which is no longer in clinical use (Fig. 3). Although rosiglitazone is significantly more potent than troglitazone as a PPARγ ligand and transactivator, rosiglitazone in turn is likely to be superseded by even more potent agents such as GW7845, which have been 'custom designed' to activate genes regulated by PPARγ (Fig. 3). A further stage in the evolution of this process will see the use of new SPARMs such as LG100641 (Ref. 30) to achieve selective organ specificity and desired selective agonist/antagonist activity. Moreover, the known synergy between SPARMs and rexinoids should enhance therapeutic use[40,42,45].

Molecular pharmacology of SPARMs

Like other class II members of the nuclear receptor superfamily, PPARγ functions as an obligate heterodimer with RXRs (Ref. 15). One consequence of heterodimerization with RXR is the ability of this heterodimer to be activated by ligands for both PPARγ and RXR. Transactivation of PPARγ target genes is a multi-step process that first involves binding of the PPARγ–RXR heterodimer to specific DR1-type response elements in the promoter of a target gene. In the absence of ligand, this heterodimer associates with a complex of co-repressor proteins that silence the promoter by deacetylating histones in the adjacent chromatin[52]. Next, ligand binding induces a conformational change in the receptor, which causes dissociation of the co-repressor complex, and permits the heterodimer to interact with at least two co-activator complexes, namely p160/CBP and DRIP (also called TRAP or ARC)[53,54]. These two complexes acetylate histones, thereby making adjacent chromatin more accessible to the interaction of the PPARγ–RXR heterodimer with putative target genes and the basal transcriptional machinery. This ligand-induced transactivation is dependent on: (1) the different types of co-repressors and co-activators associated with the receptor heterodimer, and (2) the relative affinities of these cofactors for PPARγ and RXR.

The ability to modulate the activity of a receptor in a tissue-specific manner by altering the strength and type of receptor-cofactor interactions is the molecular basis for the selective activity of SERMs and similar modulators[26]. It comes as no surprise that one pharmacologic action of a SPARM might be to facilitate the interaction of a specific cofactor with the PPARγ–RXR heterodimer and thereby selectively alter transcriptional activity of PPARγ. Indeed, it has already been shown that RXR and PPARγ agonists recruit different co-activators to the heterodimer[55]. Thus, the tissue-specific PPARγ co-activator, PGC-1 (Refs 56,57), is a relevant therapeutic target in Syndrome X and other obesity-related disorders. Clearly, an important goal of future research will be to determine the expression of such cofactors (both co-activators and co-repressors) during carcinogenesis.

In addition to their direct, ligand-dependent effects on transcription, ligands for PPARγ are also known to antagonize signal transduction pathways by mechanisms independent of this receptor[58]. Perhaps the best characterized of these effects is the general antagonism of PPARγ agonists on inflammation. Both TZDs and the natural agonist prostaglandin 15d-PGJ_2 have been reported to inhibit the production of inflammatory cytokines and inducible nitric oxide synthase (iNOS) (Refs 59–61), and reduce inflammation typically found in colitis[62]. Attenuation of inflammatory signaling pathways by PPARγ agonists has been attributed in part to inhibition of nuclear factor (NF)-κB signaling, suggesting that a ligand-dependent, protein-protein interaction occurs between PPARγ and one of the components of the NF-κB signaling

cascade[61]. Because the mechanisms by which PPARγ mediates ligand-dependent transactivation and inhibition of NF-κB are clearly different, one could speculate that SPARMs might be developed that can select between the two mechanisms.

Conclusions

The striking new data on PPARγ and its ligands now offer an exciting prospect for the prevention and treatment of malignancy. Many new approaches can be pursued, and here we suggest only a few. Clearly, the synthesis and testing of new and more selective SPARMs will be of primary importance. The opportunity to combine the use of new and selective SPARMs with other new and selective receptor modulators, such as RXR modulators (or even SERMs), offers a particularly intriguing possibility, in that new and unforeseen properties might emerge from the selective interactions with co-activators and co-repressors that might result from liganded PPARγ–RXR heterodimers[55]. Moreover, it has recently been suggested that some ligands for PPARγ might also mediate some of their effects, particularly the suppression of inflammation, through pathways independent of this receptor[58].

Studies on the interface of SPARMs and PPARγ with other multifunctional cellular regulatory systems, such as the TGF-β signalling pathway (reviewed in Ref. 63) and the NF-κB signalling pathway (reviewed in Ref. 64) will allow integration of PPARγ studies with many other areas of cell biology, such as inflammation, regulation of the cell cycle, apoptosis, invasion, and metastasis, that are highly relevant to carcinogenesis. Recent studies have indicated already that PPARγ has direct interactions with both the TGF-β (Ref. 65) and NF-κB (Refs 61,66) pathways. Yet another area that needs to be investigated is the possibility of using SPARMs in combination with agents that modify chromatin structure, to make genes more responsive to inducers of differentiation. Agents that affect histone acetylation[67,68] or DNA methylation[69,70] can make tumor cells more responsive to a differentiating agent such as all-*trans*-retinoic acid, and such a strategy should be investigated for potentiation of the activity of SPARMs. This approach might be particularly useful for the prevention or treatment of ER-negative breast cancer, given that ER-negative cells are believed to retain a functional PPARγ system[46], even when regulation by estrogen is lost.

Hopefully, advances in the molecular and cellular biology, as well as the pharmacology, of the PPARγ system can be readily translated to clinical applications. The use of SPARMs to prevent or treat cancer represents only one of many clinical challenges. There are important possibilities in many other areas of clinical medicine. These include diabetes[29], atherosclerosis[71], and inflammatory bowel diseases such as Crohn's disease and ulcerative colitis[62]. Advances in all of these areas have been reported already, and the known ability of PPARγ to regulate genes for proteins that are directly relevant not only to cancer, but also to the above diseases (such as resistin, CD36, SR-A, LXR-α, matrix metalloprotease 9, iNOS, and the tumor suppressor PTEN) indicates that there is a strong mechanistic basis for such clinical advances[43,58,60,72–75]. Studies on the role of SPARMs in the prevention and treatment of cancer could have both theoretical and practical implications for the prevention and treatment of an even wider range of human diseases. At the same time, a note of caution is required, as there might be undesirable side effects, presently unforeseen, that emerge from chronic administration of ligands for a multifunctional receptor such as PPARγ.

Acknowledgements
This work was supported by the National Foundation for Cancer Research, the Oliver and Jennie Donaldson Trust, NIH Grant R01 CA78814, the Dept of Defense (DAMD17-99-1-9168 and DAMD17-98-1-8604), and the Howard Hughes Medical Institute.
We thank Millard Lambert for Figure 2, and Dominic Klyve for expert assistance in the preparation of this review. M.B.S. is the Oscar M. Cohn Professor.

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Pergamon

Bioorganic & Medicinal Chemistry Letters 12 (2002) 1027–1030

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

A Novel Dicyanotriterpenoid, 2-Cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile, Active at Picomolar Concentrations for Inhibition of Nitric Oxide Production

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Received 14 November 2001; accepted 31 January 2002

Abstract—New oleanane triterpenoids with various substituents at the C-17 position of 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) and methyl 2-carboxy-3,12-dioxooleana-1,9(11)-dien-28-oate were synthesized. Among them, 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile shows extremely high inhibitory activity ($IC_{50}=1$ pM level) against production of nitric oxide induced by interferon-γ in mouse macrophages. This potency is about 100 times and 30 times more potent than CDDO and dexamethasone, respectively. © 2002 Elsevier Science Ltd. All rights reserved.

In previous papers, we reported that 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oic acid (CDDO) (**1**), its methyl ester **2** and methyl 2-carboxy-3,12-dioxo-oleana-1,9(11)-dien-28-oate (**3**) show high inhibitory activity against production of nitric oxide (NO) induced by interferon-γ (IFN-γ) in mouse macrophages ($IC_{50}=0.1$ nM level).[1–4] We also reported that CDDO is a potent, multifunctional agent in various in vitro assays.[5] For example, CDDO induces monocytic differentiation of human myeloid leukemia cells and adipogenic differentiation of mouse 3T3-L1 fibroblasts. CDDO also inhibits proliferation of many human tumor cell lines, and blocks de novo synthesis of inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (COX-2) in mouse macrophages. The above potencies have been found at concentrations ranging from 10^{-6} to 10^{-9} M in cell culture. Mechanism studies revealed that CDDO is a ligand for peroxisome proliferator-activated receptor γ (PPARγ)[6] and induces apoptosis in human myeloid leukemia cells.[7]

Modifications of rings A and C of oleanolic acid (**30**), a commercially available naturally occurring triterpene, led to the synthesis of CDDO. However, we had not modified the carboxyl group at C-17 of CDDO, which is very important from the perspective of structure–activity relationships (SARs). Because the synthesis of CDDO involves 11 steps from oleanolic acid, this has limited the preparation of sufficient quantities of CDDO to allow such modifications. However, we have recently produced a sufficient amount to be able to synthesize various CDDO derivatives with modified carboxyl groups (i.e., nitrile, esters, glycosides, and amides) at C-17 (see Table 1). As a result, we found that 2-cyano-3,12-dioxooleana-1,9(11)-dien-28-onitrile (**4**) shows extremely high inhibitory activity ($IC_{50}=1$ pM level) against production of NO in mouse macrophages. This potency is about 100 times and 30 times more potent than that of CDDO and dexamethasone, respectively. In this communication, we report the synthesis, inhibitory activity and SARs of these new analogues.

Dinitrile **4** was synthesized from CDDO by the method as shown in Scheme 1. Addition of oxalyl chloride to CDDO gave acyl chloride **31** in quantitative yield. Amide **15** was prepared in 91% yield from **31** with ammonia gas in benzene. Dehydration of **15** with thionyl chloride gave **4** in 89% yield.[8] Because the C-17 carboxyl group of CDDO is hindered, esterifications of CDDO with alcohols under acidic conditions were not successful. We found that a nucleophilic substitution

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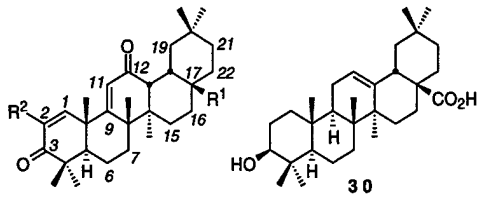
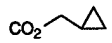
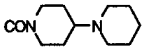
PII: S0960-894X(02)00105-1

Table 1. Synthesis and biological potency of new oleanane triterpenoids



30

Compd	R^1	R^2	Method	Yield (%) from 1	IC_{50} (nM)[a]
CDDO (1)	CO_2H	CN	Refs 1 and 4		0.44
2	CO_2Me	CN	Refs 1 and 4		0.11
3	CO_2Me	CO_2H	Refs 2 and 4		9.55
4	CN	CN	Scheme 1	81	0.0035
5	CN	CO_2H	Scheme 3		1.68
6	CO_2Et	CN	A	100	0.80
7	CO_2Et	CO_2H	Scheme 3		7.93
8	$CO_2CH_2CH{=}CH_2$	CN	B	83	1.33
9	$CO_2(CH_2)_3CH_3$	CN	A	74	6.65
10		CN	A	81	4.45
11	CO_2CH_2Ph	CN	A	97	4.35
12	$CO_2(CH_2)_7CH_3$	CN	A	89	60.4
13	CO-D-Glu(OAc)$_4$	CN	Scheme 2	75	0.070
14	CO-D-Glu	CN	Scheme 2	62	10.1
15	$CONH_2$	CN	Scheme 1	91	0.098
16	$CONHNH_2$	CN	C	55	0.26
17	CONHMe	CN	D	93	0.58
18	$CONH(CH_2)_2CH_3$	CN	D	93	1.50
19	$CONH(CH_2)_5CH_3$	CN	D	92	14.9
20	CONHPh	CN	D	100	28.6
21	$CONHCH_2Ph$	CN	D	96	9.2
22	$CONMe_2$	CN	D	89	1.55
23	CON(*n*-Pr)$_2$	CN	D	85	32.9
24		CN	E	86	0.80
25		CN	E	66	0.95
26		CN	E	82	1.00
27		CN	E	59	2.40
28		CN	C	83	0.014
29		CN	C	92	12.0
30	Oleanolic acid				>40,000
	Dexamethasone				0.10

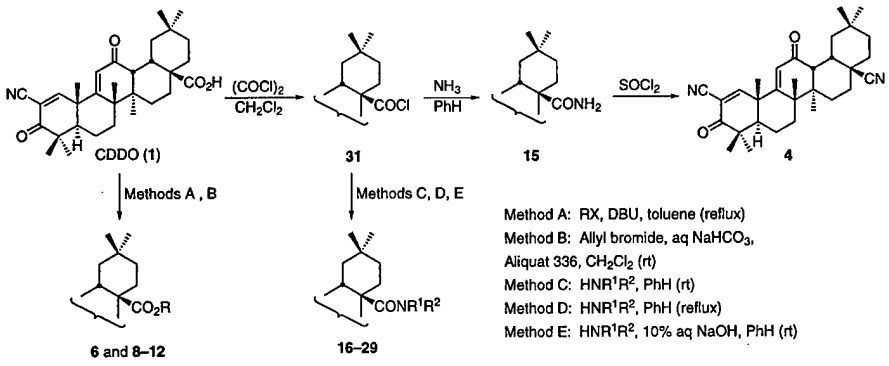
[a]IC_{50} values of compounds **1–29** and dexamethasone were determined in the range of 0.01 pM–1 μM (10-fold dilutions). Values are an average of several separate experiments. None of the compounds was toxic to primary mouse macrophages at 1 μM.

method using an alkyl halide and DBU in toluene (reflux)[9] gives esters **6** and **9–12** from CDDO in good yield (see Table 1). Allyl ester **8** was successfully prepared in 83% yield from allyl bromide and CDDO using a phase-transfer catalyst.[10] Amides **16–29** were synthesized in good yield by condensation reactions (Methods C and D, see Scheme 1) between acyl chloride **31** and the corresponding amines. Tetra-*O*-acetyl-β-D-glucopyranoside **13** was prepared in 75% yield from tetra-*O*-acetyl-α-D-glucopyranosyl bromide[11] and CDDO using a phase-transfer catalyst.[12] Because in the ^1H NMR spectrum (300 MHz, $CDCl_3$) of **13** the anomeric proton was observed at δ 5.70 ppm (1H, d, $J = 7.8$ Hz), the proton was assigned the β-configuration. Acetyl groups of **13** were removed with saturated ammonia methanol solution to afford β-D-glucopyranoside **14** in 83% yield (Scheme 2). In addition to these CDDO derivatives, we have synthesized derivatives of compound **3**, nitrile **5** and ethyl ester **7** (Scheme 3). Their syntheses require many more steps than the syntheses of CDDO derivatives because the carboxyl group at C-2 must be introduced after the carboxyl group at C-17 is modified. Acid **33** was prepared in 83% yield by cleavage of the known methyl ester **32**[1,4] with LiI in DMF.[13] The same sequence as for **4** gave nitrile **34** in 25% yield (chlorination, 100%; amidation, 100%; and dehydration, 25%). The desired nitrile **5** was synthesized in 4 steps from **34** (yield, 24%) according to the known synthetic sequence for **3**[2,4] (insertion of carboxyl group at C-2 of **34** with Stiles' reagent,[14] followed by methylation with diazomethane, 48%; insertion of double bond at C-1 with phenylselenenyl chloride–pyridine and subsequent H_2O_2 oxidation,[15] followed by selective hydrolysis of the C-2 methyl ester with KOH in aqueous methanol, 51%). Ethyl ester **35** was prepared in 99% yield by ethyl iodide and DBU in toluene. The desired ethyl ester **7** was synthesized in 57% yield from **35** by the same sequence as for **5**.

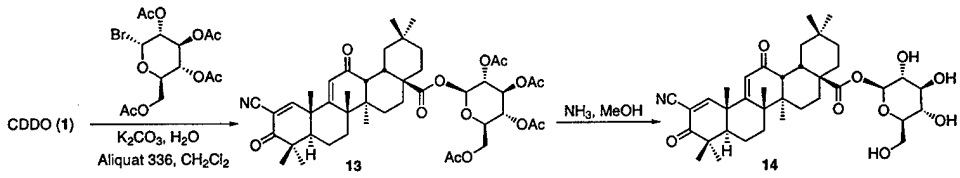
The inhibitory activities [IC_{50} (nM) value] of new synthetic triterpenoids **4–29**,[16] oleanolic acid, and dexamethasone on NO production induced by IFN-γ in mouse macrophages[17] are shown in Table 1. Dinitrile **4** shows extremely high potency ($IC_{50} = 1$ pM level); it is about 100 times and 30 times more potent than CDDO and dexamethasone, respectively.

These results provide the following SARs about substituents at C-17: (1) A nitrile group enhances potency. Dinitrile **4** is much more potent than **1** and **2**, nitrile **5** is more potent than **3**. (2) Ester moieties decrease potency. The less polar the ester, the less is its potency. Ester **12** is much less potent than **1** and **2**. (3) Tetra-*O*-acetyl-D-glucopyranoside **13** is more potent than **1** and **2**. D-Glucopyranoside **14** is much less potent than **1**, **2**, and **13**. Interestingly, in this case, the more polar the compound, the less is its potency. However, because we have only one example, we cannot conclude that this will be a general relationship. (4) Amide moieties decrease potency, although amide **15** and hydrazide **16** show similar potency to those of **1** and **2**. The less polar the amide, the less is its potency. (5) Although carbonyl imidazole **28** is about 30 times more potent than **1**, because this moiety is much more reactive than the other moieties with nucleophiles, it is difficult to compare it with the other moieties. Interestingly, the carbonyl pyrazole **29**, with less reactivity than **28**, is much less potent than **1** and **28**.

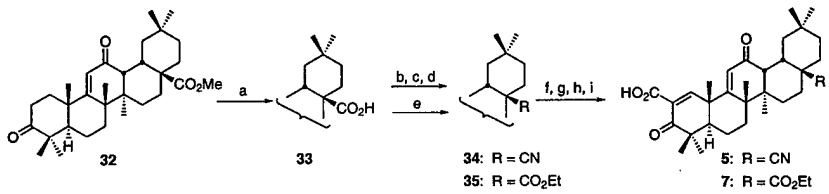
Some of these compounds including **4** had good in vivo antiinflammatory activity, when given ip or po, against peritoneal inflammation induced by thioglycollate and



Scheme 1.



Scheme 2.



Scheme 3. (a) LiI, DMF; (b) $(COCl)_2$, CH_2Cl_2; (c) NH_3, PhH; (d) $SOCl_2$; (e) EtI, DBU, toluene; (f) Stiles' reagent, DMF; (g) CH_2N_2, Et_2O, THF; (h) PhSeCl, pyr, CH_2Cl_2; 30% H_2O_2, CH_2Cl_2; (i) KOH, aq MeOH.

IFN-γ. We will report these data elsewhere. Further biological evaluation of dinitrile **4** is also in progress.

Acknowledgements

We thank Drs. Carl Nathan and Qiao-wen Xie for expert advice on the preparation of macrophages and the nitric oxide assay. We also thank Dr. Steven Mullen (University of Illinois) for the mass spectra. This investigation was supported by funds from NIH Grant 1 R01-CA78814, US Dept. of Defense Grants DAMD17-96-1-6163, DAMD17-98-1-8604, DAMD17-99-1-9168, the Oliver and Jennie Donaldson Charitable Trust, the National Foundation for Cancer Research, and a Zenith Award from the Alzheimer's Association. M. B. S. is an Oscar M. Cohn Professor. F. G. F., Jr. is an Oscar M. Cohn Scholar.

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16. All new compounds **4–29** exhibited satisfactory spectral data including high-resolution mass spectra and elemental analyses. Dinitrile **4**: amorphous solid; $[\alpha]_D^{25}$ +21° (*c* 0.29, $CHCl_3$). UV (EtOH) λ_{max} (log ε) 244 (4.30) nm. IR (KBr) 2947, 2871, 2253, 2233, 1690, 1666 cm^{-1}. ^1H NMR ($CDCl_3$) δ 8.04 (1H, s), 6.01 (1H, s), 3.26 (1H, d, *J*=4.8 Hz), 2.78 (1H, ddd, *J*=3.3, 4.8, 13.5 Hz), 1.55, 1.53, 1.26, 1.18, 1.01, 1.00, 0.91 (each 3H, s). ^{13}C NMR ($CDCl_3$) δ 197.9, 196.6, 169.4, 165.6, 125.0, 123.9, 114.9, 114.5, 50.1, 47.9, 46.1, 45.2, 42.8, 42.3, 38.4, 35.1, 34.2, 33.7, 33.3, 32.5, 31.9, 30.7, 28.2, 27.2, 26.9, 25.2, 23.9, 23.1, 21.8, 21.7, 18.4. EIMS (70 eV) *m/z* 491 $[M]^+$ (100), 472 (29), 457 (14), 269 (100). HREIMS calcd for $C_{31}H_{40}N_2O_2$: 472.3090. Found: 472.3095. Anal. calcd for $C_{31}H_{40}N_2O_2 \cdot H_2O$ C, 75.88; H, 8.63; N, 5.71. Found: C, 75.53; H, 8.58; N, 5.69.
17. Briefly, the procedure for this assay is as follows: Macrophages were harvested from female mice injected intraperitoneally four days previously with 4% thioglycollate. These cells were seeded in 96-well tissue culture plates and incubated with 4 ng/mL IFN-γ in the presence or absence of inhibitory test compounds. After 48 h NO production (measured as nitrite by the Griess reaction) was determined. Full details of the assay are given in ref 18.
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OPINION

Chemoprevention: an essential approach to controlling cancer

Michael B. Sporn and Nanjoo Suh

Mortality that results from the common forms of cancer is still unacceptably high. Despite immense advances in the understanding of the mechanisms of carcinogenesis, in bringing potent new drugs to the clinic and in treating several relatively rare forms of cancer, overall mortality statistics are unlikely to change in a fundamental way until there has been a re-orientation of emphasis in cancer research that will direct greater resources towards prevention of new disease, rather than treatment of end-stage disease.

The failure to control cancer deaths from common epithelial malignancies — such as those that occur in the lung, breast, prostate, colon, pancreas and ovary (TABLE 1) — provides the ultimate rationale for an approach based on prevention. Although chemotherapy of advanced disease has been highly successful for the treatment of relatively rare cancers — such as those of the testis, childhood leukaemias and Hodgkin's disease — the prognosis for patients with invasive and metastatic disease at the common epithelial sites (which account for more than half the cancer deaths in men and women) remains poor. Chemotherapy of earlier stages of disease, when invasion is still local, has been more successful, but it is always possible that some dissemination of malignancy might have occurred when therapy is started. The logical approach to controlling cancer is to prevent it before the complex series of genetic and epigenetic events that result in invasive and metastatic malignancy have occurred. Chemoprevention — which uses pharmacological agents to impede, arrest or reverse carcinogenesis at its earliest stages — is one way of accomplishing this[1].

Chemoprevention has been successfully achieved in numerous animal experiments over the past 25 years, and has been validated in several major clinical trials (see REFS 2,3 for reviews). Sceptics have asserted that this approach is not truly 'prevention', but rather suppression or delay of an undesirable outcome[4]. However, this criticism only serves to divert attention from the practical goal of decreasing the incidence of invasive cancer and death from cancer at an early age. Prevention need not be absolute (many important vaccines are effective only for limited periods of time) — it encompasses hindering the occurrence of an undesirable outcome, as well as precluding it altogether[5]. Extension of the latency period of carcinogenesis[1,4] — so that people can have a high quality of life before dying of another cause at an advanced age — is a highly desirable strategy for controlling cancer and extending lifespan, even if total cure of advanced malignancy cannot be achieved.

Chemoprevention

A mechanistic approach. Successful implementation of chemoprevention depends on a mechanistic understanding of carcinogenesis at the molecular, cellular and tissue levels. If carcinogenesis is considered as a disease of aberrant differentiation — which is almost axiomatic at the present time; see the classic reviews by Pierce *et al.*[6] and Mintz and Fleischman[7] — then the potential role that the 2,000–3,000 proteins that regulate gene transcription might have in this process must also be considered[8], as differentiation is ultimately determined by the expression of specific genes. As well as gene mutation, there is strong evidence that dysfunctional epigenetic control — such as aberrant methylation of DNA or acetylation of histones — is also associated with carcinogenesis[9–11]. Similarly, in addition to the transcription factors themselves, defects in the complex signal-transduction cascades that regulate the activity of these regulators can also contribute to the carcinogenic process.

Carcinogenesis, however, is more than the result of having too many cells; it is the result of too many aggressive, invasive cells that are in the wrong place at the wrong time. So, although molecular lesions in genes that regulate the cell cycle might enhance carcinogenesis, the tumorigenic process also affects the normal relationships between epithelial cells and their underlying stromal cells[12–14]. Carcinogenesis can therefore be redefined as a disease of tissue, which involves several cell types. The end result — invasive and metastatic carcinoma — is the final stage of many dysfunctional steps at both the cellular and tissue level. This complex process will probably require many pharmacological agents to prevent end-stage disease. At present, it seems unlikely that a single 'magic bullet' will ever be found that can either prevent carcinoma or treat metastatic malignancy.

Pharmacology of chemoprevention

The most rational approach to chemoprevention is to design and test new agents that act on specific molecular and cellular targets. In addition, it is essential that the efficacy and safety of new agents are validated in experimental models before clinical trials are begun. Although epidemiological studies can provide valuable leads for the development of chemopreventive agents, they need to be

Table 1 | **Total estimated cancer deaths in the United States**

Site	1971	2002
Total cancer deaths in men		
Lung	53,000	89,000
Colorectal	22,000	28,000
Prostate	17,000	30,000
Pancreas	10,000	15,000
Total cancer deaths in women		
Lung	11,000	66,000
Colorectal	24,000	29,000
Breast	31,000	40,000
Pancreas	8,000	15,000
Ovary	10,000	14,000

Numbers are from American Cancer Society statistics (see REFS 69,70, which provide more details). They have not been adjusted for age or population; the latter has increased by ~40% during the past 30 years. These data are not intended to imply that there have not been significant advances in the management of carcinoma at all of the above sites, but rather to emphasize the crucial need for new approaches to its control.

confirmed with experimental data in cell culture and animal models before clinical trials are initiated.

Empirical approaches to chemoprevention — based solely on epidemiological data that correlate dietary patterns and risk of developing cancer — are fraught with danger, as was dramatically seen in the clinical failure of the trials that used β-carotene to prevent cancer at common sites such as the lung, prostate and colon[15,16]. The selection of β-carotene for these trials was based largely on data indicating that consumption of large amounts of fruits and vegetables — in which β-carotene is found — was associated with a reduced risk of developing cancer, but there were almost no data in experimental animals to indicate that β-carotene itself prevented cancer at the above sites. Fortunately, many new agents are now available that target molecular and cellular processes that are known to be important in carcinogenesis; these agents also prevent cancer in experimental animals (see REFS 1,2,17 for comprehensive surveys). So, which of them show particular promise?

Nuclear-receptor ligands. Members of the nuclear-receptor superfamily are transcription factors that selectively regulate cell differentiation and proliferation in specific organs — many of which are important sites for carcinogenesis[18] — making their ligands an ideal target for chemoprevention. Nuclear receptors that are involved in carcinogenesis and have been targeted for chemoprevention include the two oestrogen receptors (ERα and ERβ), the androgen receptor, the three retinoic acid receptors (RARα, β and γ), the three retinoid X receptors (RXRα, β and γ), the vitamin D receptor (VDR) and peroxisome proliferator-activated receptor-γ (PPARγ) (TABLE 2).

Transcriptional activity of these receptors is controlled by the binding of specific ligands, which can activate transcription in one cellular context, but also repress transcription in another. Although this phenomenon of contextual action was puzzling when originally discovered, it is now understood in terms of the selective recruitment and displacement of other proteins — transcriptional co-activators and co-repressors — that interact with the transcription factors themselves[19]. So, tamoxifen should no longer be viewed as an 'oestrogen antagonist'; it is a selective oestrogen-receptor modulator (SERM) that can be anti-oestrogenic in one organ, such as the breast, and pro-oestrogenic in others, such as bone and the uterus[20,21]. Anti-oestrogenic activity of tamoxifen in the breast is desirable, as oestrogen enhances the growth of almost all breast cancer cells during early stages of carcinogenesis; by contrast, stimulation of uterine epithelium by tamoxifen can potentially cause endometrial carcinoma. For chronic administration of a SERM for breast cancer prevention, it is also important that it is pro-oestrogenic in bone, to avoid osteoporosis that results from oestrogen deprivation.

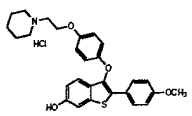
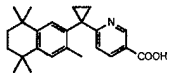
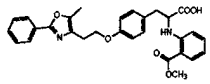
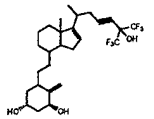
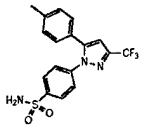
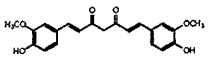
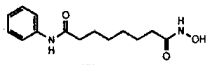
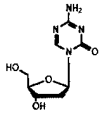
The SERM concept — and its extension to other members of the nuclear-receptor superfamily that are also selective modulators; for example, selective PPARγ modulators (SPARMs)[22] — has great importance for the entire field of chemoprevention, because a single modulator drug that can have the total spectrum of desired actions (both agonistic and antagonistic) in different organs of the body is now within reach. Although this goal has not yet been achieved, the development of newer SERMs, such as raloxifene and arzoxifene (which are anti-oestrogenic in breast and uterus, and pro-oestrogenic in bone), indicates that it will be possible to achieve further benefits of SERMs while eliminating some of the undesirable actions of older agents[21,23,24].

Chemopreventive benefits of SERMs might not be limited to actions in the breast, as the recent identification of ERα and ERβ in the prostate indicates that they could provide a useful target for prevention of prostate cancer[25]. Indeed, prevention of prostate cancer with tamoxifen — which binds to both ERα and ERβ — was shown in a rat model several years ago[26]. Similarly, oestrogen receptors that are present in the colon also have the potential to modulate carcinogenesis in that organ[27]. However, so far, clinical efficacy of SERMs for cancer prevention has been shown only in the breast for tamoxifen[20] and raloxifene[23].

Rexinoids — ligands that are selective for binding to any or all of the three RXRs — are another group of highly promising chemopreventive agents. As RXRs form functional heterodimers with many other nuclear receptors — including the RARs, VDR and PPARγ — rexinoids have the unique ability to modulate the actions of many transcription factors[28]. They have already proven useful as single agents in animal models, for preventing both ER-positive[29] and ER-negative[30] breast cancer. Prevention of ER-negative cancer is a particularly important achievement, as these malignancies generally have a poor prognosis. Moreover, although RXRs are not known to interact directly with ERs, the combination of the rexinoid LGD-1069 and tamoxifen has unusual potency, not only in preventing, but also in treating breast cancer in rats[29].

The synthetic analogues of vitamin D ('deltanoids'), many of which have potent differentiative and anti-proliferative activities[31,32], are yet another set of promising ligands for chemoprevention. Numerous epidemiological studies indicate an inverse association between vitamin-D intake and human cancer risk, especially in the colon[33], and new laboratory findings show that the VDR can protect the colon from the carcinogenic effects of bile acids[34]. However, ingestion of increased amounts of natural forms of vitamin D is dangerous because of the resultant increase in calcium levels in the blood (hypercalcaemia). The development of new synthetic deltanoids, which are strongly anti-proliferative but have a much lesser propensity to cause hypercalcaemia, has been an important advance[31,32]. The VDR is a

Table 2 | **Chemopreventive agents with known molecular targets**

		Molecular target	Successful use of this class for prevention in animals	Clinical trials of this class of agent
Ligands for nuclear receptors				
Arzoxifene (SERM)		Oestrogen receptors	Breast cancer, prostate cancer	Successful (breast)
LG100268 (Rexinoid)		Retinoid X receptors	Breast cancer	Planned
GW7845 (SPARM)		Peroxisome proliferator-activated receptor-γ	Breast cancer	Planned
Ro24-5531 (Deltanoid)		Vitamin D receptor	Breast cancer, colon cancer prostate cancer	Planned
Anti-inflammatory agents				
Celecoxib		Cyclooxygenase-2	Colon cancer	Successful
Curcumin		Nuclear factor κB	Breast cancer, colon cancer	Planned
Chromatin modifiers				
SAHA		Histone deacetylase	Breast cancer	In progress
5-Aza-2′-deoxycytidine		DNA (demethylating agent)	Colon cancer, lung cancer	Drug too toxic at present

Structures of chemopreventive agents with known molecular targets. The ligands for the four nuclear receptors are all synthetic analogues of naturally occurring hormones or metabolites that bind to their cognate receptors. Celecoxib has been designed for specific inhibition of cyclooxygenase-2 (COX-2), in contrast to non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit both COX1 and COX2. Curcumin is representative of several dietary constituents that have anti-inflammatory and anti-carcinogenic activities, and that are mediated by suppression of the activity of the transcription factor, nuclear factor κB. Suberoylanilide hydroxamic acid (SAHA) is a prototype of an entire series of hydroxamic acids that have been synthesized as histone deacetylase inhibitors, and is in clinical trial. 5-Aza-2′-deoxycytidine is the prototypical DNA demethylating agent. Its practical use for chemoprevention is limited by its toxicity, and development of safer demethylating agents is an important goal. Both classes of chromatin modifiers can potentiate the actions of other drugs, especially ligands for nuclear receptors, by making specific genes more receptive to transcriptional activation (see REFS 9–11,36,43,44 for specific examples and reviews). These potentiating actions of chromatin modifiers emphasize the importance of the use of drug combinations for practical implementation of chemoprevention[61]. Chemical structures shown are only single examples of entire classes of agents. SERM, selective oestrogen-receptor modulator; SPARM, selective peroxisome proliferator-activated receptor-γ modulator.

classic transcription factor and has been shown to interact functionally with SMAD3, a component of the signal-transduction pathway for the regulatory cytokine transforming growth factor-β (TGF-β)[35].

So, ligands for the nuclear-receptor superfamily show complex interactions: they control gene expression directly, and they interact with a much larger set of regulatory pathways[8,36] by cross-talk with signal-transduction cascades. With progress in the synthesis of new deltanoids that are almost totally non-calcaemic, it seems that VDR ligands will be increasingly important. There are abundant data to indicate that they are highly active for prevention in animal models[32], but clinical proof is yet to come.

Anti-inflammatory agents. The concept that inflammation and carcinogenesis are causally linked has been proposed for more than a century, but it has recently taken on a new importance as anti-inflammatory agents that inhibit the formation of prostaglandins have been shown to be useful in chemoprevention (TABLE 2). Non-steroidal anti-inflammatory drugs (NSAIDs), and especially the newer selective inhibitors of cyclooxygenase-2 (COX2) such as celecoxib, inhibit colon carcinogenesis in experimental animals[37], and cause a significant reduction in the number of colorectal polyps in human subjects[38]. Mechanistically, these studies are particularly interesting as the beneficial effects of COX2 inhibitors seem to be mediated by their effects on stromal cells of the intestine, especially their ability to suppress angiogenesis, which is part of the stromal reaction (reviewed in REF. 39).

In addition to agents that act directly as inhibitors of COX2, the transcription factor nuclear factor κB (NF-κB), which regulates the activities of many genes that are involved in the inflammatory process, provides an excellent target for the development of new chemopreventive agents. For example, several natural products that have both anti-inflammatory and anti-carcinogenic activity — such as curcumin, resveratrol and caffeic acid phenethylester (CAPE) — block either the activation or transcriptional activity of NF-κB[40]. The development of new inhibitors

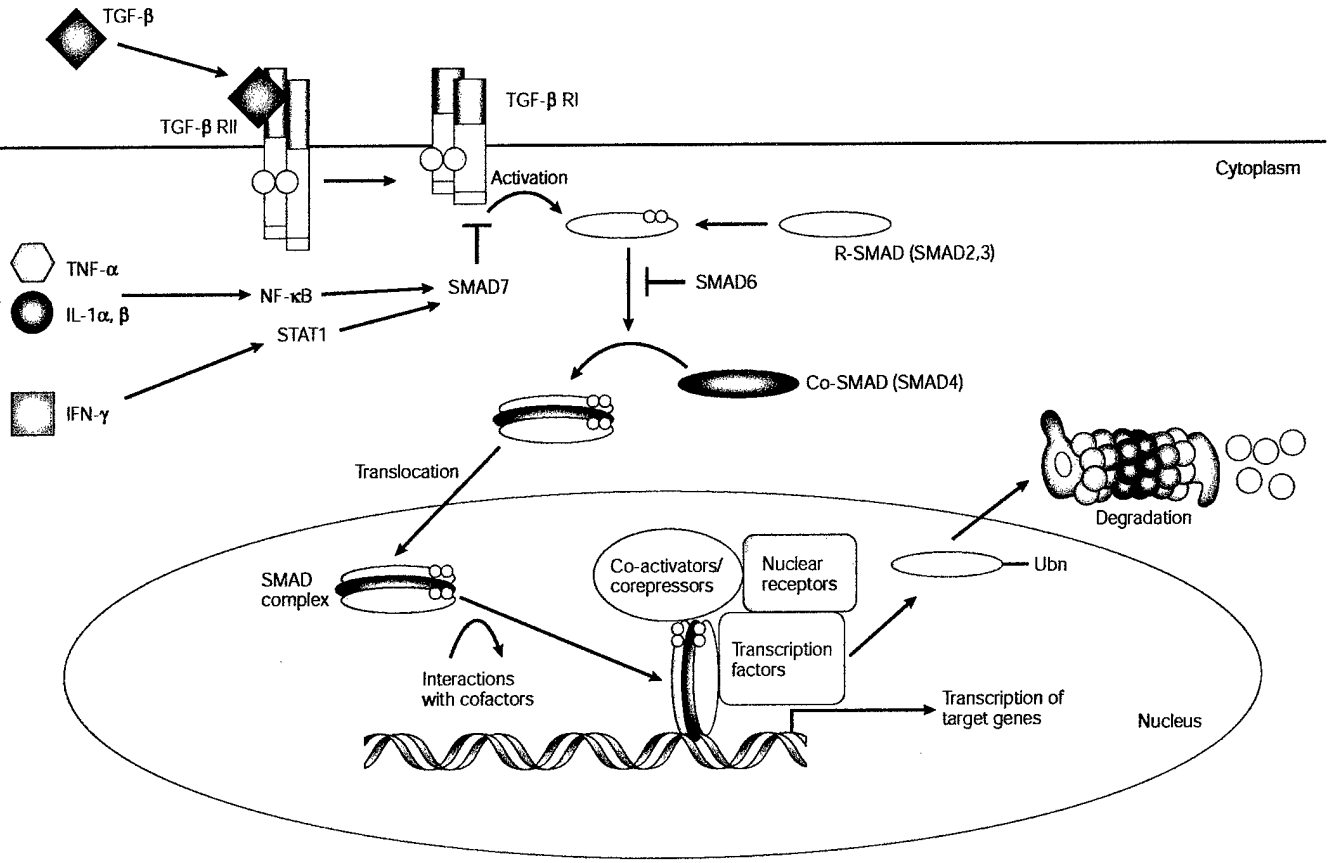


Figure 1 | **The TGF-β signalling pathway represents a new target for potential development of new drugs for chemoprevention.** This greatly oversimplified diagram (see REFS 46–48 for detailed reviews) shows several molecular targets that could be usefully modulated. SMAD 2, 3 and 4 activate gene transcription after appropriate phosphorylation (shown as light brown circles in both the receptors and SMADs) and translocation to the nucleus. Interactions of SMADs with co-activators and corepressors (which can modulate chromatin structure by altering acetylation of histones), in turn, modulate transcriptional activation. Functional interactions of SMAD3 with several nuclear receptors, including the vitamin-D receptor[35], the glucocorticoid receptor[62], the oestrogen receptors[63], the androgen receptor[64,65] and peroxisome proliferator-activated receptor-γ (PPARγ)[66] have been reported, and again indicate that combinations of agents that affect SMAD signalling, together with ligands for nuclear receptors, might result in synergistic action for chemoprevention. The three cytokines tumour-necrosis factor-α (TNF-α), interleukin-1α (IL-1α) and interferon-γ (IFN-γ), which are crucial mediators of inflammation, are shown because of the importance of the inflammatory process as a promoter of carcinogenesis. The signalling pathways of these cytokines (involving the transcription factors nuclear factor κB (NF-κB) and signal transducer and activator of transcription 1 (STAT1)) can upregulate the inhibitory SMAD7[67,68] to suppress transforming growth factor-β (TGF-β) signalling. TGF-β itself can also induce both SMAD7 and the inhibitory SMAD6 in a negative regulatory loop (not shown). SMADs are also regulated by ubiquitin (Ubn)-mediated degradation, and this pathway is also a potential target for drug development (see REFS 8,36,46–48 for detailed reviews). RI and RII, receptor 1 and 2, respectively.

of NF-κB is being widely pursued for treatment of diseases other than cancer (for example, inflammatory bowel disease or neurodegenerative disease) and illustrates the diverse applications of studies of regulation of gene transcription in modern pharmacology. However, suppression of NF-κB activity is not without risk, as it can increase susceptibility to infections such as tuberculosis.

Chromatin modifiers. A new area of research that has started to impact on chemoprevention is the study of chromatin modifiers, which can alter the activities of many other agents that are ligands either for transcription factors or for other proteins that modulate the activities of transcription factors. Chromatin structure can be modified with drugs that either increase the acetylation of histones (histone deacetylase inhibitors) or demethylate cytosine residues in DNA[9–11,36], and thereby increase the ability of transcription factors to stimulate gene expression. The two classes of agents are interactive[41], and members of both classes have been successfully used for chemoprevention in experimental animals[9,42] (TABLE 2). Mechanistically, chromatin modifiers interact with ligands for the nuclear-receptor superfamily, as can be seen in the ability of trichostatin A (a histone deacetylase inhibitor) or 5-aza-2′-deoxycytidine (a DNA demethylating agent) to enhance the ability of all-*trans*-retinoic acid either to differentiate human leukaemia cells[43] or to suppress the proliferation of human breast cancer cells[44]. The practical use of many chromatin modifiers has been limited so far by their toxicity, but the development of new, less toxic, agents, such as suberoylanilide hydroxamic acid (SAHA)[11], indicates that this problem can be solved.

Control of signal transduction

The past 10 years have seen heightened activity in studies of signal-transduction pathways, which has resulted in the development of important new molecular therapeutics, such as selective tyrosine kinase inhibitors for chronic myelogenous leukaemia (imatinib; Gleevec) and anti-ERBB2 monoclonal antibodies for breast cancer (trastuzumab; Herceptin). It is inevitable that this approach will have important implications for chemoprevention of cancer, particularly as many

inhibitors of signal transduction seem to be relatively free of the undesirable toxicities of classical chemotherapeutic agents.

A good example of a prime target for the development of new chemopreventive agents is the TGF-β serine–threonine kinase signalling pathway (FIG. 1). The cytokine TGF-β negatively regulates epithelial-cell growth, and its loss of function has been implicated in many areas of carcinogenesis[45–48]. It is well established that inactivating mutations of the type II TGF-β receptor are a frequent occurrence in human gastric and colon cancer; furthermore, transcriptional repression of the gene that encodes this receptor is also very common in human cancer cells[49]. Most recently, defects in type I receptors have been implicated in the genesis of ovarian carcinoma[50]. Loss of TGF-β signalling during carcinogenesis could also occur within the cell as a result of mutations in the SMAD pathway (see FIG. 1), which mediates the activity of the ligand–receptor complex to regulate gene expression. So, a drug that enhanced the signalling activity of the SMAD2–SMAD3–SMAD4 complex would have the potential to bypass defects in the upstream receptor complex, and a drug that blocked the inhibitory activity of SMAD7 would also have the potential to enhance TGF-β signalling. Interestingly, hyperactivity of SMAD7 has been clinically implicated in inflammatory bowel disease, which can enhance risk for colon cancer[51,52]. A cautionary note here is that enhancing TGF-β signalling can contribute to invasion and metastasis in advanced stages of carcinogenesis[46–48].

Beyond this speculative discussion of the development of new chemopreventive drugs that interact with SMADs is the reality of using chemotherapeutic agents that have already been developed to target other signal-transduction cascades, such as the epidermal growth factor (EGF)/ERBB2 receptor signalling cascades[53,54], as such drugs might also be useful for chemoprevention. Furthermore, signal-transduction cascades converge on many transcription factors (such as NF-κB[55] and the STAT (signal transducer and activator of transcription) family[56,57]), or other regulatory molecules such as the cyclins and their respective kinases[58]. The individual activities of all these regulators of cell function might represent potentially useful targets for new preventive agents.

Importance of animal experiments

Showing the efficacy of chemopreventive agents in appropriate animal models is essential before they can enter into clinical trials. As noted above, failure to observe this caveat with β-carotene has had unfortunate consequences in the past. We do not wish to imply that epidemiological studies cannot provide important leads for further clinical studies of chemopreventive agents, but such information should be reinforced with laboratory data before extremely expensive and time-consuming clinical trials are begun. Fortunately, excellent rodent models of breast, prostate, lung and colon cancer exist[59]. The development of transgenic mice for carcinogenesis studies has been an important advance and now offers the possibility of studying the prevention of ER-negative breast cancer[30], in addition to the well-established rat models for ER-positive breast cancer. A typical design and the results of a chemoprevention study are shown in FIGS 2,3. These figures illustrate the sophistry of suggesting that chemopreventive agents merely 'delay' the onset of cancer but do not 'prevent' it. Most chemoprevention experiments in experimental animals are performed in groups of animals that have either been treated with a massive dose of carcinogen or that have a genetic defect that results in most of the control animals developing malignant tumours in a short time period (FIG. 2). This use of animals with overwhelming carcinogenic burden is not a particularly valid model of human carcinogenesis, but rather a reflection of the practical and economic considerations that enable an investigator to evaluate a preventive agent in a reasonable time and at a reasonable cost in the laboratory setting.

It is easy to evaluate a preventive agent with a relatively small number of animals when the controls have 100% tumour incidence within a short latency period, and this is a preferred method for laboratory studies. However, in humans the latency period for carcinogenesis is long[3] (10–20 years, or even more at most sites), and, moreover, incidence of invasive cancer is fortunately low. Most heavy smokers do not develop invasive lung cancer, even though all probably have genetic lesions and cellular abnormalities in their bronchial epithelium. So, when very high doses of carcinogen are used in chemopreventive studies, it might seem that a preventive agent is only delaying the onset of cancer. However, when more relevant low doses of carcinogen are used, suppression of the onset of cancer over very long time periods can be shown (FIG. 3). The important concept to emphasize is that an effective chemopreventive agent can extend the latency period for onset of cancer, often more than doubling this parameter, so that an animal might never develop a visibly invasive carcinoma. Chemopreventive agents that double the latency period for human carcinogenesis would have an immense impact on raising the quality of life for millions of people.

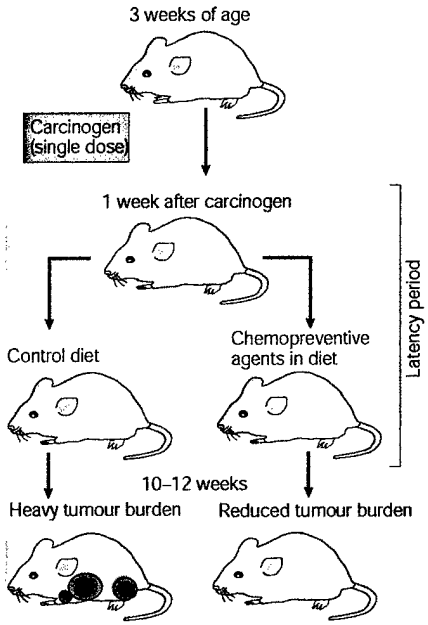


Figure 2 | **A standard study design for evaluating a new chemopreventive agent.** In the example shown, the rat model uses nitrosomethylurea as a carcinogen[59]. The importance of the latency period is discussed in the text and shown diagrammatically in FIG. 3.

Controversies in chemoprevention

Chemoprevention of cancer, in contrast to chemoprevention of cardiovascular disease with statins or anti-hypertensive agents, is still a highly controversial topic. One of the primary misperceptions that hinders the adoption of preventive measures for cancer is the common notion that people are 'healthy' until they are told they have an invasive cancer[1–3]. Failure to recognize risk at the earliest stages of disease (when the disease process could be controlled) causes failure to adopt sensible preventive measures. At the same time, there is a realistic concern that a preventive agent should be as free from deleterious side effects as possible.

So, safety is a paramount issue, as the level of safety that is required for the use of a preventive agent is markedly greater than that required for a classical therapeutic agent. So, how do we decide who should take a new preventive agent that might have even minor side effects? Two important criteria for this decision are the level of risk of the prospective patient and the inadequacy of alternatives other than chemoprevention. So, in extremely high-risk populations — such as in women who have a *BRCA1* or

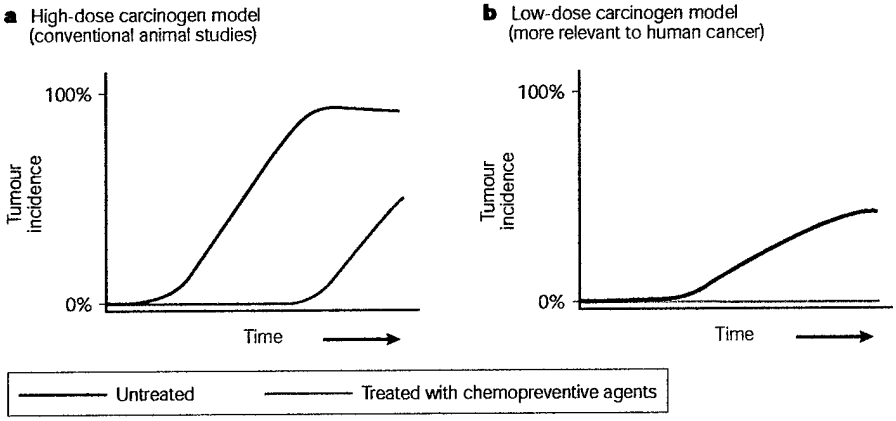


Figure 3 | **Extension of the latency period is important for chemoprevention in both animals and humans. a** | If carcinogenic burden in an animal model is artefactually high, then it might seem that a chemopreventive agent might only delay the appearance of a cancer. **b** | However, if such carcinogenic burden is reduced to levels that are more relevant to human carcinogenesis, a more meaningful extension of latency can be obtained, which would allow a lifetime suppression of malignancy.

BRCA2 mutation and so are likely to develop breast cancer, or in patients with familial adenomatous polyposis who are almost certain to develop colon cancer (for which the therapeutic alternatives, such as prophylactic mastectomy or total removal of the colon, respectively, are also highly undesirable) — it is relatively easy to justify a trial of a new chemopreventive agent.

However, in other people with lower risk, or who have more acceptable alternatives, more difficult decisions are faced. As more data on efficacy and safety become available from chemoprevention trials in high-risk groups, it will be easier to justify the use of chemopreventives in populations at lower risk. Furthermore, the development of new and better biomarkers of efficacy[3] will allow the evaluation of new agents over shorter time periods, again allowing the testing of many more agents with potentially greater safety margins[3]. Ultimately, the ethical clinical use of any preventive agent depends on a benefit-versus-risk analysis[2]. It will be essential to develop even safer chemopreventive agents if this approach is to be widely adopted in human populations at relatively low risk, although the use of chemopreventive agents in groups at exceptionally high risk is clearly justified at present.

Summary

We have not dealt with the cancer problem during the past century in a realistic way, as we have not allocated the resources or effort that are necessary to prevent this disease. Although treatment of end-stage disease remains a clinical necessity, we should redirect more of our efforts to place a greater emphasis on prevention of disease and less emphasis on magic bullets of cure. Chemoprevention of epithelial carcinogenesis now offers a realistic, practical approach to controlling common forms of cancer in men and women. Chemoprevention is a clinically proven modality in the areas of head and neck[60], breast[20,23] and colon[38] carcinogenesis, and has been shown to prevent cancer in almost every common target site in experimental animals[2,3,17]. However, there is still an overwhelming need to develop even more effective and safer chemopreventive agents and to use them in synergistic combinations. Development of 'combination chemoprevention' will be essential[61], just as combination chemotherapy has been so important in the treatment of invasive disease. Ultimately, prevention is a quality-of-life issue. Prevention of disease in its earliest stages offers a higher quality of life than treatment of end-stage disease.

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doi:10.1038/nrc844

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Acknowledgements

We thank M. Padgett for expert assistance in the preparation of the manuscript. Our research is supported by grants from the National Cancer Institute, the Department of Defense, the National Foundation for Cancer Research, and the Oliver and Jennie Donaldson Trust. M. B. S is an Oscar M. Cohn Professor.

Online links

DATABASES

The following terms in this article are linked online to:

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breast cancer | chronic myelogenous leukaemia | colon cancer | endometrial cancer | gastric cancer | head and neck cancer | Hodgkin's disease | lung cancer | ovarian cancer | pancreatic cancer | prostate cancer | testicular cancer

LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
androgen receptor | *BRCA1* | *BRCA2* | COX1 | COX2 | EGF | ERBB2 | ERα | ERβ | IFN-γ | IL-1α | NF-κB | PPARγ | RARα | RARβ | RARγ | RXRα | RXRβ | RXRγ | SMAD2 | SMAD3 | SMAD4 | SMAD6 | SMAD7 | STAT1 | TGF-β | TNF-α | VDR

Medscape DrugInfo:
http://promini.medscape.com/drugdb/search.asp
celecoxib | Gleevec | Herceptin | raloxifene | tamoxifen

OMIM: http://www.ncbi.nlm.nih.gov/Omim/
familial adenomatous polyposis | inflammatory bowel disease | osteoporosis

SCIENCE AND SOCIETY

Cancer after nuclear fallout: lessons from the Chernobyl accident

Dillwyn Williams

The Chernobyl accident exposed people located hundreds of kilometres away to fallout, but increases in cancer incidence as a result of the accident seem, at present, to be restricted to one tumour type. These thyroid tumours form the largest number of cancers of one type, caused by a single event on one date, ever recorded. Epidemiological, pathological and molecular studies have provided new insights into the carcinogenic process, as well as lessons for future nuclear accidents.

Four years after the Chernobyl accident, unusual numbers of thyroid cancer cases in children were noted in Belarus and Ukraine[1], and were reported to the International Atomic Energy Agency (IAEA). These reports were greeted sceptically in the West, partly because many felt that isotopes of iodine carried relatively little carcinogenic risk, and partly because it was considered that the latency period was far too short. Fifteen years on, what have we learned about the consequences of this, the largest ever peacetime nuclear accident?

The accident took place on 26 April 1986, when a misguided experiment went disastrously wrong. A steam explosion blew the lid off the reactor and a fire in the graphite core led to the release of over 10^{19} becquerels (Bqs) of radioisotopes, including nearly all of the volatile isotopes in the reactor, with high levels of fallout over Belarus, Northern Ukraine and part of the Russian Federation (FIG. 1). Some 10–20 million people were exposed to significant levels of fallout; several hundred workers at the plant received whole-body radiation, 134 developed acute radiation sickness and, of these, 28 died within four months[2]. But what long-term effects did the accident have?

Thyroid cancer incidence

The first official reports, in 1992, described 114 cases of thyroid cancer in children in Belarus during a 30-month period; the increase started in 1990, less than four years after the accident[3,4]. By comparison, there were only two cases in 1986, rising to six in 1989. The diagnoses were confirmed in 102 of 104 cases examined. These were not incidental microcarcinomas, but clinically significant